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Award Number: DAMD17-02-1-0612

TITLE: A Novel Apoptosis Pathway that is Defective in Early Breast Cancer

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REPORT DATE: April 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Final(1 Apr 2002 - 31 Mar 2005)

4. TITLE AND SUBTITLE
A Novel Apoptosis Pathway that is Defective in Early Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0612

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This grant was awarded to Dr. A. Thorburn but transferred to Dr. S. Cramer when Dr. Thorburn left Wake Forest University in Sept. 2004. The project examines a novel cell death pathway that is induced by the death domain of the adaptor protein FADDD (FADD-DD). FADD-DD-induced cell death occurs only in normal primary epithelial cells (from the breast or prostate) and can be inhibited when epithelial cells are immortalized e.g. by the expression of the telomerase catalytic subunit and the large and small T/t antigens from SV40 virus. The objectives of the project were to determine if T/t antigens cause resistance on their own and to determine how they do so. We found that Large T antigen was sufficient to confer selective resistance to this cell death pathway but that this resistance was not caused by the known activities of T antigen that are involved in immortalization including p53 inactivation or Rb inactivation. Moreover, we were able to expand our studies beyond that proposed in the grant and showed that the FADD-DD cell death pathway occurs in genetically-defined mouse epithelial cells and is selectively inactivated by spontaneous immortalization and that the cell death pathway involves both apoptosis and autophagy.

14. SUBJECT TERMS
apoptosis, signal transduction, tumorigenesis, oncogenes, transformation

15. NUMBER OF PAGES
54

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION.

Evasion of apoptosis is a hallmark of cancer [1]. Consequently, it is sometimes thought that cancer cells are generally resistant to apoptosis while normal cells are sensitive. In fact cancer cells are actually closer to their apoptotic threshold than their normal counterparts and therefore often undergo apoptosis more easily in response to diverse apoptotic stimuli [2]. This apoptosis sensitization occurs because growth promoting oncogenic events such as Myc expression raise the levels of caspases and other apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. However, it is not clear if this is the only apoptotic barrier that cancer cells must overcome as they become transformed. Are there also specific apoptosis pathways that inhibit cancer development and are active in normal cells and specifically inactivated during tumor development? We hypothesized that such a pathway would have the unusual characteristic of working in normal cells but not in cancer cells. Furthermore unlike most apoptotic stimuli, which usually work better in cancer cells than their normal counterparts because they are closer to their apoptotic threshold, signaling proteins and physiological stimuli that activate this kind of pathway should induce specific apoptosis responses in normal cells that should be selectively inhibited in cancer cells without affecting responsiveness to other apoptotic stimuli. The apoptosis pathway we study that is induced by FADD-DD has these characteristics because it works in normal epithelial cells but does not work in immortalized epithelial cells. Resistance to this pathway can be achieved by expression of SV40 Large T antigen. The established mechanism of FADD-induced apoptosis involves activation of caspase-8, however we previously showed that our pathway does not involve caspase-8 indicating that this is a novel mechanism of FADD-dependent apoptosis. Furthermore, as outlined in our previous annual report (April 2003), the resistance to this apoptosis pathway that arises in immortal cells is specific in the sense that it does not affect other apoptotic pathways including those induced by a FADD molecule that can activate caspase-8. The goals of this project were: 1) To determine whether T/t antigen alone can confer resistance and 2) To determine why T/t antigen causes resistance. These experiments were intended to determine why breast cancer cells are resistant to this apoptosis pathway

BODY.

As outlined in the previous annual reports and in the appended publication (Thorburn et. al. 2005), the proposed tasks were successfully completed and we were able to significantly expand this work to achieve much more than we originally had hoped. The approved statement of work for this project had the following goals.

Task 1. Determine whether T/t antigens alone are responsible for resistance to FADD-DN-induced apoptosis (months 1-12)

Task 2. Determine why expression of T/t antigens causes resistance of breast cancer cells to FADD-DN-induced apoptosis (months 12-36)

Task 1 was completed in year 1 according to plan. We demonstrated that only Large T antigen was necessary for conferring resistance to FADD-DD-induced cell death and that both small t antigen and TERT were not involved. The bulk of our work has been towards the goals in aim 2. Because Large T antigen inactivates p53, the most likely explanation for this result was that the cell death pathway is p53-dependent. However a series of experiments indicated that this is not the case. The most definitive way of showing this came from studies that were prompted by the suggestions of a reviewer of our first annual report. These experiments showed primary breast epithelial cells from p53 knockout animals were equally sensitive to FADD-DD-induced cell death but that upon continued culture in vitro, the cells became selectively resistant to the death pathway without displaying altered resistance to other cell death stimuli. We therefore next asked in T antigen conferred

resistance through inactivation of the retinoblastoma gene product. Again, we found that Rb deficiency did not alter the FADD-DD-induced cell death of primary breast epithelial cells. To determine if combined deficiency of both p53 and Rb pathways was responsible, we used breast cells that are deficient in the INK4a and ARF genes and again showed that while these primary cells were sensitive to FADD-DD the same cells became resistant upon continued culture in vitro. Detailed description of the experiments using both human and mouse breast cells and confirming our results in human prostate cells is presented in an appended paper [3].

Additional Research Achievements.

We were also able to extend our work beyond that originally proposed in the grant application in several important directions. First, we were able to ask if a physiological stimulus could activate the FADD-DD pathway in normal epithelial cells. Using both normal epithelial cells [3, 4], we found that activation of TRAIL receptors leads to induction of the FADD-DD pathway through the endogenous FADD protein. Importantly resistance to this cell death pathway occurred even in cells that were still sensitive to the canonical FADD-dependent apoptosis pathway that works through caspase-8 [3, 4]. These results are important because they demonstrate that the FADD-DD signaling pathway can be stimulated through the endogenous FADD protein but that the pathway that is activated is mechanistically distinct from the canonical death receptor signaling pathway. We extended these studies to look at the requirements for FADD activation by TRAIL receptors and identified mutant FADD proteins (and mutant receptors) that prevent FADD activation by TRAIL receptors [5, 6]. One such mutant (a point mutation of valine 108 in the FADD–DD) was used in a series of experiments, which showed that this mutant could prevent TRAIL-induced activation of the FADD-DD pathway in normal breast cells [3].

Our data indicate that the FADD-DD-induced cell death mechanisms involves both caspase-dependent apoptosis (which works through caspase-9 not caspase-8 [4], and another form of cell death. We were able to demonstrate that this cell death mechanism is associated with autophagic vesicle formation and can be blocked by autophagy inhibitors [3]. This result is exciting for several reasons. First, recent work from other groups has shown that regulators of autophagy are intimately involved in breast (and others) cancer development. For example, the autophagy regulator beclin 1 is a haploinsufficient tumor suppressor and mice that lack one allele of this gene get breast cancer and other tumors [7, 8]. Second, work from Joan Brugge and her colleagues demonstrated that autophagy contributes to breast epithelial cell death during acinar formation and that inhibition of this death caused accumulation of cells that resemble the early stages of ductal carcinoma in situ (DCIS) [9]. Moreover, Brugge and colleagues found that the breast epithelial cell death during acinar formation can be regulated by TRAIL receptors [9]. Third, very recent work [10] suggests that the FADD DD may interact directly with an autophagy regulator called ATG5. These results are consistent with our data and suggest that defects in TRAIL-dependent apoptosis and autophagy working through the FADD-DD pathway may be involved in the earliest stages of breast cancer development.

Future Directions.

This project has provided a firm basis for the continued expansion of these studies, which is the subject of ongoing research in the Thorburn lab. Our efforts are focused on further characterization of the FADD-DD pathway, exploration of the mechanism of immortalization-dependent resistance, and examination of the molecular mechanism of autophagy induction. This work is proceeding in both human and mouse breast epithelial cells and is supported by a new NIH RO1 that was awarded March 2005. Because the work supported by the grant also led us to explore the role of the TRAIL pathway in breast cancer, we have been able to expand our work to directly address the role of this pathway in the development, progression and treatment of breast

cancer leading to new research projects that are being developed in the lab that will examine TRAIL pathway defects in a cohort of 315 breast cancer patients.

KEY RESEARCH ACCOMPLISHMENTS.

We achieved our goals set out in the approved statement of work and were able to extend our studies to make inroads into other areas including the role of TRAIL receptor signaling in this response and the role of non-apoptotic cell death via autophagy.

We showed that FADD-DD-induced cell death occurs only in non-immortalized breast epithelial cells.

We showed that resistance to FADD-DD-induced cell death is highly selective- occurring without affecting sensitivity to other cell death pathways, including the canonical pathway that is activated by FADD through caspase-8.

We showed that although immortal epithelial cells are resistant to FADD-DD-induced cell death, this resistance is not accomplished by the known activities that are involved in immortalization including activation of telomerase activity, inactivation of p53, inactivation of pRB or inactivation of both the p53 and RB pathways via INK4a and ARF.

We showed that the FADD-DD pathway could be activated by a physiological stimulus (TRAIL receptor activation) and that the FADD-DD pathway could be blocked by a FADD point mutant that was identified in a study of TRAIL receptor-FADD interactions.

We showed that the FADD-DD pathway involves both caspase-dependent apoptosis, which works through caspase-9 and autophagy and that these two cell death mechanisms occur in parallel.

This pathway therefore represents the first example that we know of a cell death response that is epithelial specific and selectively inactivated upon cell immortalization.

REPORTABLE OUTCOMES.

The following publications were supported by the grant. The primary research articles directly address the tasks outlined in the statement of work and work that arose from the extension of these studies (e.g. to examine TRAIL signaling in normal epithelial cells and the TRAIL-FADD interaction). In addition two review articles that describe work supported by this grant are listed.

Thorburn, J., Bender, L. M., Morgan, M. J., and Thorburn, A. (2003). "Caspase- and serine protease-dependent apoptosis by the death domain of FADD in normal epithelial cells." *Mol. Biol. Of the Cell.* **14,** 67-77.

Thorburn, A. (2004). "Death receptor-induced cell killing." *Cellular Signalling.* **16,** 139-144.

Thomas, L.R., Henson, A. Reed, J.C., Salsbury, F. R., and Thorburn, A. (2004). "Direct binding of FADD to the TRAIL receptor DR5 is regulated by the death effector domain of FADD."*J. Biol. Chem.* **279,** 32780-32785.

Thomas, L.R., Reed, J.C., Johnson, R. and Thorburn, A. (2004) "The C-terminal Tails of Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors have opposing functions in Fas Associated Death Domain (FADD) recruitment and can regulate agonist-specific mechanisms of receptor activation." *J. Biol. Chem.* **279**. 52479-52486.

Thorburn, J., Moore, F., Rao, A, Barclay, W., Thomas, L.R., Grant, K.W, Cramer, S.D. and Thorburn, A. (2005) "Selective inactivation of a FADD-dependent apoptosis and autophagy pathway in immortal epithelial cells." *Mol. Biol. Of the Cell.* **16,** 1189-1199.

CONCLUSIONS.

The work supported by this grant therefore achieved the original goals, led to unanticipated discoveries particularly about TRAIL signaling and autophagy during the earliest stages of breast cancer development and has formed a strong basis for expanded research in these areas in the future that is now supported by other funding through NCI.

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Molecular Biology of the Cell
Vol. 14, 67–77, January 2003

Caspase- and Serine Protease-dependent Apoptosis by the Death Domain of FADD in Normal Epithelial Cells[V]

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Submitted April 17, 2002; Revised August 21, 2002; Accepted September 30, 2002
Monitoring Editor: Martin Raff

The adapter protein FADD consists of two protein interaction domains: a death domain and a death effector domain. The death domain binds to activated death receptors such as Fas, whereas the death effector domain binds to procaspase 8. An FADD mutant, which consists of only the death domain (FADD-DD), inhibits death receptor–induced apoptosis. FADD-DD can also activate a mechanistically distinct, cell type–specific apoptotic pathway that kills normal but not cancerous prostate epithelial cells. Here, we show that this apoptosis occurs through activation of caspases 9, 3, 6, and 7 and a serine protease. Simultaneous inhibition of caspases and serine proteases prevents FADD-DD–induced death. Inhibition of either pathway alone does not prevent cell death but does affect the morphology of the dying cells. Normal prostate epithelial cells require both the caspase and serine protease inhibitors to efficiently prevent apoptosis in response to TRAIL. In contrast, the serine protease inhibitor does not affect TRAIL-induced death in prostate tumor cells suggesting that the FADD-DD–dependent pathway can be activated by TRAIL. This apoptosis pathway is activated in a cell type–specific manner that is defective in cancer cells, suggesting that this pathway may be targeted during cancer development.

INTRODUCTION

Apoptotic caspases can be separated into "initiator caspases" such as caspase 8 and 9 that start an apoptotic cascade and "effector caspases" such as caspase 3, 6 and 7 that disassemble the cell (Nicholson, 1999). Two main pathways leading to caspase activation have been characterized (Hengartner, 2000). The extrinsic pathway is activated by ligand-bound death receptors of the tumor necrosis factor (TNF) receptor family (Ashkenazi and Dixit, 1998). The six identified death receptors contain an intracellular protein interaction domain called a death domain and induce apoptosis by forming a complex (called the DISC) at the death domain. The adapter FADD is an essential component of the DISC (Chinnaiyan *et al.*, 1995). FADD consists of two protein interaction domains: a death domain and a death effector domain that interacts with a death effector domain on procaspase 8. FADD binds to the receptor or other adapters such as TRADD (Hsu *et al.*, 1995) through interactions between death domains to recruit caspase 8 to the DISC. Aggregation of initiator caspases at the DISC leads to their autoactivation (Salvesen and Dixit, 1999), and they in turn activate effector caspases causing the cell to undergo apoptosis. Because FADD is an essential component of the DISC, a mutant (FADD-DD, also called FADD-DN) that consists of the death domain, but no death effector domain has been widely used to determine whether FADD signaling is required for apoptosis. FADD-DD acts as an inhibitor because it competes with the wild-type protein and binds to activated receptors but cannot recruit and activate

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02–04–0207. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.E02–04–0207.

[V] Online version of this article contains video material. Online version is available at www.molbiolcell.org.

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Abbreviations used: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; AIF, apoptosis-inducing factor; CFP, cyan fluorescent protein; DISC, death-inducing signaling complex; Dox, doxycycline; dn8, dominant-negative caspase 8; dn9, dominant-negative caspase 9; FADD, Fas-associated death domain protein; FADD-DD, FADD-death domain; FRET, fluorescence resonance energy transfer; PARP, polyADP ribose polymerase; TNF, tumor necrosis factor; TRADD, TNF receptor–associated death domain protein; TRAIL, TNF-related apoptosis inducing ligand; YFP, yellow fluorescent protein; zIETD.fmk, benzoylcarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; zVAD.fmk, benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone.

caspase 8. FADD-DD inhibits apoptosis by all death ligands (Wajant *et al.*, 1998) and several other stimuli.

Diverse stress pathways cause release of mitochondrial proteins into the cytosol to activate the other apoptosis pathway—the intrinsic pathway. Protein release occurs after binding of proapoptotic Bcl-2 family members and other proteins, e.g., the transcription factor TR3 (Li *et al.*, 2000), to mitochondria. Antiapoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xL inhibit mitochondrial protein release to prevent apoptosis. Cytoplasmic cytochrome c (cyt c) interacts with Apaf-1, procaspase 9, and dATP to form a complex called the apoptosome (Li *et al.*, 1997). This complex activates caspase 9, which then activates effector caspases to induce apoptosis. Other proapoptotic mitochondrial proteins include apoptosis-inducing factor (AIF; Susin *et al.*, 1999), Smac/Diablo (Du *et al.*, 2000; Verhagen *et al.*, 2000), endonuclease G (Li *et al.*, 2001), and Omi/HtrA2 (Suzuki *et al.*, 2001; Hegde *et al.*, 2002; Martins *et al.*, 2002; Verhagen *et al.*, 2002). Death receptors can activate the intrinsic pathway through cleavage of Bid (Luo *et al.*, 1998).

Other proteases in addition to caspases are also involved in apoptosis (Johnson, 2000; Leist and Jaattela, 2001a). For example, several studies implicate lysosomal proteases (cathepsins) in apoptosis (Jones *et al.*, 1998; Guicciardi *et al.*, 2000; Foghsgaard *et al.*, 2001; Leist and Jaattela, 2001b). The spectrum of proteases that are activated in response to a stimulus affects the commitment to and the phenotype of cell death (Leist and Jaattela, 2001a).

Recently, we identified an unusual proapoptotic activity for the FADD death domain (Morgan *et al.*, 2001). Expression of FADD-DD from microinjected expression plasmids activated caspases and induced apoptosis in normal but not cancerous prostate epithelial cells. FADD-DD–induced apoptosis did not occur in normal prostate fibroblasts or smooth muscle cells, indicating that the effect is cell type specific. Despite the increased caspase activity in FADD-DD–expressing normal prostate cells, caspase inhibitors did not prevent cell death (Morgan *et al.*, 2001). These results raise several questions. First, which caspases are activated by FADD-DD? Second, which caspase activation pathway (intrinsic or extrinsic) is used? Third, do the activated caspases actually have a role in this apoptosis response? Fourth, what is the nature of the signal that kills cells when the caspases are inhibited? In this article, we answer these questions.

MATERIALS AND METHODS

Cell Culture, Microinjection, and Adenovirus Infection

Normal human prostate epithelial cells were isolated from tissue samples or obtained from Clonetics (La Jolla, CA) and cultured as previously described (Morgan *et al.*, 2001). Prostate cell lines were obtained from ATCC (Rockville, MD). Microinjection experiments were performed as previously described (Morgan *et al.*, 2001); cells were injected with expression plasmids using an Eppendorf microinjector (Newport, RI). Quantitative cell survival experiments were performed by identifying injected fluorescent cells 3 h after injection and then determining the fate of each cell (i.e., whether it lived or died) after an additional 18-h incubation. Data presented in the histograms represents the mean ± SEM from between 3 and 10 separate injection experiments with different preparations of cells and plasmids. Each experiment involved 50–200 injected cells per sample. Recombinant doxycycline (Dox)-regulated YFP and YFP-FADD-DD adenoviruses were made using the AdenoX Tet-off kit from Clontech (Palo Alto, CA). Viruses were produced according to the manufacturer's instructions and coinfection with a Tet repressor virus was performed into prostate epithelial cells. Greater than 90% infection was achieved in both tumor cell lines and normal primary prostate epithelial cells. Repression was achieved by maintaining the cells in 1 μg/ml doxycycline, and gene expression was stimulated by removal of Dox. Where indicated, cells were treated with the general caspase inhibitor zVAD.fmk (0.1 mM; Alexis, San Diego, CA), the serine protease inhibitor AEBSF (0.3 mM; Sigma, St. Louis, MO) or the caspase 8 inhibitor zIETD.fmk (0.1 mM; Calbiochem, La Jolla, CA).

Western Blotting

For Western blot analysis of caspase cleavage, cells were harvested 24–48 h after adenovirus infection. Protein samples were separated by SDS-PAGE and probed with the following antibodies: anti-YFP (Clontech); anticaspase-cleaved cytokeratin 18 (Roche, Indianapolis, IN); anti-PARP, anticaspase 8, antiactive caspase 9, anticaspase 3, antiactive caspase 7, and anticaspase 6 (Cell Signaling, Beverly, MA); and antiactin (Sigma).

Reverse Two-hybrid Screen

Reverse two-hybrid screening was performed as previously described (Thomas *et al.*, 2002). A library of >500,000 random FADD mutants was generated by mutagenic PCR and screened to identify point mutants that cannot bind to caspase 8 (a catalytically inactive mutant with cysteine 360 in the active site mutated to alanine was used) but retain the ability to bind to Fas as well as the wild-type protein. Between 1 and 23 separate isolates of the following single-point mutants were identified in the screen. Leu 7 to Pro, Leu 8 to Pro, Ser 10 to Pro, Ser 12 to Leu, Ser 12 to Pro, Ser 13 to Pro, Leu 15 to Pro, Leu 23 to Pro, Leu 23 to Arg, Leu 26 to Pro, Leu 49 to Pro, Leu 55 to Pro. The mutations to proline are likely to disrupt alpha helices in the DED. Mutants L8P, S12L, and L15P were chosen for further analysis, expressed as GFP-tagged fusion proteins and used for cell injection experiments.

Fluorescence Resonance Energy Transfer Assays of Caspase Activity

FRET assays for caspase activation were performed as previously described (Morgan and Thorburn, 2001) except that the blue fluorescent protein-yellow fluorescent protein described previously was replaced with a cyan-yellow fusion. The caspase-cleavable linker peptide was identical to the previous fusion protein. Cells were injected with the FRET construct along with FADD-DD expression plasmid (without a fluorescent tag) and Bcl-xL expression plasmid or empty vector then maintained in an environmental chamber at 37°C and 5% CO_2 on a Zeiss Axiovert 100 microscope (Thornwood, NY). The following images were captured at 30-min intervals: phase, FRET (excite cyan at 440 nm, detect yellow emission at 575 nm), cyan (excite cyan at 440 nm, detect cyan emission at 485 nm). For each cell the ratio of yellow/cyan fluorescence per unit area was calculated for each time point after subtraction of the background fluorescence as previously described (Morgan and Thorburn, 2001). Quantitation was halted when the injected cells began to contract as determined by the total area of the cell being reduced by half. There was no consistent difference in the time that control and Bcl-xL–expressing cells began to contract. The increase in caspase activity was calculated as the inverse of the percent change in yellow/blue fluorescence ratio for each cell. Quantitation for 120 min before cell rounding is shown to provide a measure of the temporal changes in caspase activation that occur in individual cells.

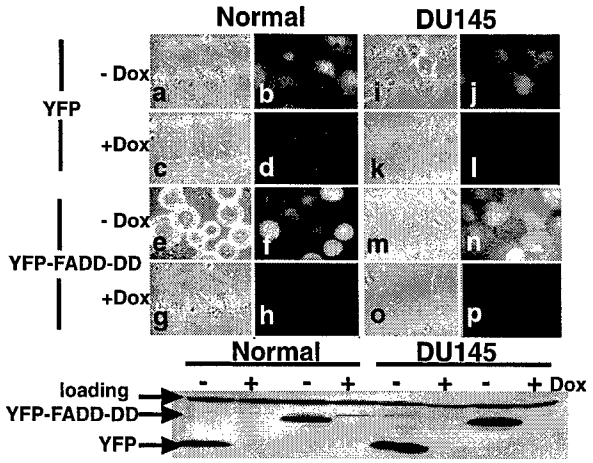

Figure 1. FADD-DD selectively kills normal prostate epithelial cells. Normal primary prostate epithelial cells or DU145 tumor cells were infected with Dox-regulated adenoviruses expressing YFP (a,b,c,d,i,j,k,l) or YFP-tagged FADD-DD (e,f,g,h,m,n,o,p) and a TetR virus. Adjacent panels show phase and fluorescence images of the same field. Expression was induced by removing Dox. FADD-DD caused normal cells to die (e and f) but did not affect DU145 cells (m and n). YFP had no effect in either cell type. The lower panel shows total protein probed with anti-YFP. Equal amounts of protein were expressed in each case. The top band is a cross-reacting protein serving as a loading control.

Time-lapse Microscopy

YFP-FADD-DD–injected cells were maintained in the environmental chamber in the presence of zVAD.fmk (0.1 mM) and/or AEBSF (0.3 mM) where indicated. Fluorescent cells were identified and fluorescent and phase images of the same fields were captured using a Hamamatsu CCD (Malvern, PA) camera run by Openlab (Improvision, Warwick, UK) software. Images were captured at 30-min intervals for up to 24 h. Fluorescence images for each time point were overlayed on the corresponding phase image, and the resulting movie was saved in Quicktime format.

TRAIL-induced Apoptosis

Normal prostate epithelial cells or DU145 prostate tumor cells were pretreated for 30 min with 0.8 μg/ml cycloheximide and then treated with 100 ng/ml recombinant human TRAIL (Calbiochem) in the presence zVAD.fmk or AEBSF as indicated. Cells were monitored by microscopy after incubation with TRAIL for 24 h.

RESULTS

Adenoviral Expression of FADD-DD Causes Apoptosis of Normal Prostate Epithelial Cells

Our previous experiments showing that FADD-DD could induce prostate epithelial cell apoptosis were performed by microinjection of FADD-DD expression plasmids (Morgan *et al.*, 2001). To exclude the remote possibility that apoptosis was dependent on the FADD-DD delivery method and to allow the use of biochemical assays, we constructed Dox-regulated adenoviruses expressing YFP-tagged FADD-DD or a YFP control. Coinfection of these viruses with a Tet repressor virus results in efficient repression in the presence of Dox and expression when Dox is removed. Figure 1 shows YFP and YFP-FADD-DD expression in normal primary prostate epithelial cells or the prostate epithelial tumor cell line DU145. Expression was tightly regulated by Dox as demonstrated by the fluorescence images and Western blot. FADD-DD but not the YFP control caused normal cells to die (compare panels a and b with e and f). FADD-DD did not kill the tumor cells (compare panels i and j with m and n). The Western blot shows that both normal and tumor cells expressed similar amounts of YFP and YFP-FADD-DD. The FADD-DD–expressing adenovirus did not kill normal primary prostate fibroblasts or other prostate cancer cell lines (LNCaP, PC3, CA-HPV7, unpublished data). These data extend our previous experiments (Morgan *et al.*, 2001) and demonstrate that the difference in response between normal prostate epithelial cells and tumor cells is not due to differences in the expression levels of FADD-DD in the different cell types.

FADD-DD Inhibits Fas-induced Apoptosis in Prostate Tumor Cells

Induction of apoptosis by FADD-DD was an unexpected finding because this molecule has been widely used as an inhibitor of death receptor–induced apoptosis. We therefore tested whether our FADD-DD molecule could inhibit Fas-induced apoptosis in prostate tumor cells. DU145 cells activate caspase 8 and undergo apoptosis when stimulated with agonistic Fas antibodies in the presence of cycloheximide (Rokhlin *et al.*, 1998). We expressed YFP or YFP-FADD-DD from the regulated adenovirus and then treated DU145 cells with anti-Fas in the presence of cycloheximide. Phase and fluorescence images of the same field were overlayed to allow examination of the morphology of YFP or YFP-FADD-DD–expressing cells after activation of Fas signaling. Figure 2A shows that FADD-DD prevented Fas-induced cell death, whereas YFP did not. The dying cells showed typical characteristics of apoptosis with multiple membrane blebs. Western blotting for the appearance of the cleaved form of caspase 8 showed that FADD-DD expression prevented activation of caspase 8 in response to Fas (Figure 2B). These data indicate that our FADD-DD molecule inhibits apoptosis signaling by activated death receptors. Therefore, the novel proapoptotic ability of FADD-DD in normal prostate epithelial cells occurs in addition to the established antiapoptotic functions of this molecule, which occur in prostate tumor cells.

FADD-DD Activates Caspases in Normal Epithelial Cells

We used adenovirus-infected cells and Western blotting to test whether caspases are activated by FADD-DD. First, we tested whether FADD-DD could cause the appearance of caspase-dependent epitopes in endogenous proteins. Cytokeratin 18 is cleaved by effector caspases (Caulin *et al.*, 1997) during epithelial cell apoptosis. A caspase-dependent neoepitope revealed by cleavage of cytokeratin 18 at Asp398 is recognized by the M30 antibody (Bantel *et al.*, 2000). The antibody recognizes two fragments of ~45 and 21 kDa, depending on whether a second caspase site at Asp 237 is also cleaved (Bantel *et al.*, 2000). To test whether FADD-DD expression led to cytokeratin 18 cleavage, we expressed YFP or YFP-FADD-DD from the regulated adenoviruses, har-

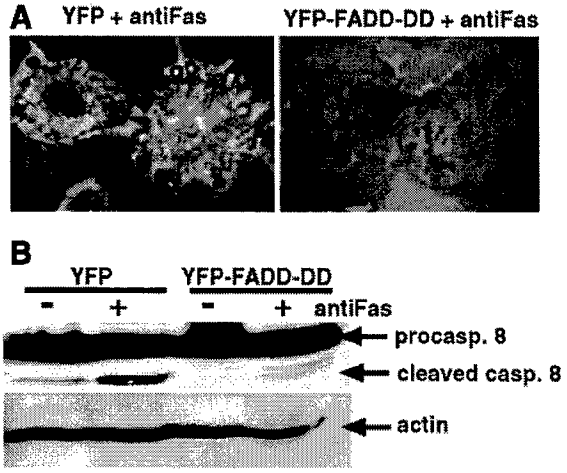

Figure 2. FADD-DD inhibits Fas-induced apoptosis in prostate tumor cells. DU145 cells were infected with the YFP or YFP-FADD-DD adenoviruses, expression was induced by removing Dox, and then cells were treated with an anti-Fas agonistic antibody. (A) Cellular morphology indicating Fas-induced apoptosis that is inhibited by YFP-FADD-DD but not by YFP. (B) Total protein samples Western blotted with anticaspase 8. Fas stimulates caspase 8 as indicated by the appearance of the processed caspase 8 band that is inhibited by YFP-FADD-DD.

vested the cells, and probed a Western blot with the M30 antibody. Figure 3A shows that FADD-DD and the positive control (sorbitol treatment to induce hyperosmolar stress-induced apoptosis) caused appearance of the 45-kDa neo-epitope, whereas YFP expression did not. Sorbitol also caused the appearance of the 21-kDa band. We also detected caspase-cleaved PARP in response to FADD-DD and sorbitol. The cleaved proteins were not present when FADD-DD–expressing cells were treated with the caspase inhibitor zVAD.fmk. These data indicate that active effector caspases are induced by FADD-DD in normal prostate cells.

We next asked which caspases were activated after expression of YFP or YFP-FADD-DD (Figure 3B). As a positive control to ensure that the antibodies worked, we used sorbitol-treated cells. Unlike the other caspases, where cleavage is required and sufficient for activation, caspase 9 does not require processing for activation (Renatus *et al.*, 2001). However, active caspase 9 digests itself when it is activated by dimerization (Renatus *et al.*, 2001). Thus, the presence of cleaved caspase 9 indicates caspase 9 activity. Sorbitol treatment and FADD-DD expression caused the appearance of active forms of caspase 9, caspase 7, and caspase 3. Active caspase 6 was detected in FADD-DD–expressing cells but not in the cells treated with sorbitol. Sorbitol and FADD-DD led to similar levels of caspase 9 cleavage but sorbitol was more effective at activating caspase 3 and caspase 7 than FADD-DD. We could not detect activation of caspase 8 by FADD-DD, however, sorbitol was effective at activating caspase 8. Activation of caspase 8 by sorbitol likely contributes to the increased activity of caspase 3 and 7 compared with FADD-DD. zVAD.fmk inhibited the appearance of cleaved forms of the caspases in FADD-DD–expressing cells, suggesting that the effector caspases are activated as a result of activation of initiator caspases (i.e., caspase 9) and

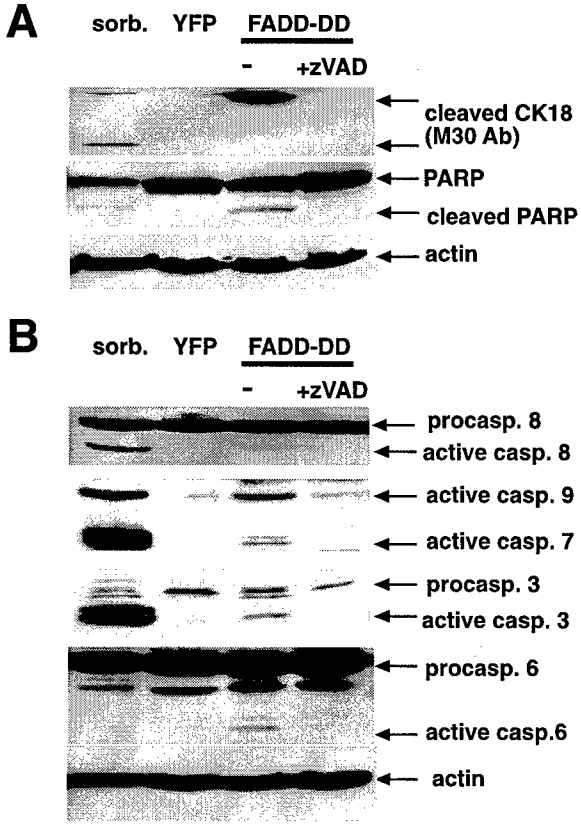

Figure 3. Caspase activation by FADD-DD. Normal prostate epithelial cells were infected with YFP or YFP-FADD-DD adenoviruses or treated with 300 mM sorbitol as a control. Cells were harvested and total protein extracts separated on gels and Western blotted with the indicated antibodies. Panel A shows that FADD-DD causes the appearance of cleaved forms of cytokeratin 18 and PARP that are blocked by treatment with zVAD.fmk. Panel B shows that active forms of caspase 9, 7, 3, and 6 but not caspase 8 are induced by FADD-DD.

that the activated caspase 9 digests itself. These data suggest that FADD-DD stimulates the intrinsic pathway in normal prostate cells to activate caspase 9 and downstream effector caspases. FADD-DD does not activate caspase 8, which is usually activated by death receptors. This is not surprising because FADD-DD lacks the death effector domain that is required for caspase 8 activation.

Caspases and Serine Proteases Contribute to FADD-DD–induced Apoptosis

Despite the fact that caspases are activated by FADD-DD, we previously found that caspase inhibitors could not prevent FADD-DD–induced prostate epithelial cell death (Morgan *et al.*, 2001). We therefore tested whether other proteases might be involved in this response. Several studies indicate a role for serine proteases in apoptosis (Johnson, 2000; Leist and Jaattela, 2001a). Furthermore, some proteins such as the tumor suppressor Bin1 induce apoptosis that can be blocked by serine protease inhibi-

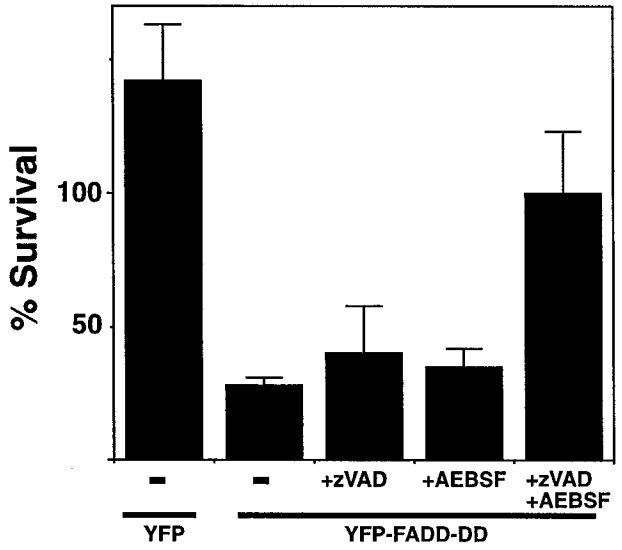

Figure 4. Caspases and serine proteases contribute to FADD-DD–induced apoptosis. Normal prostate cells were injected with the YFP control of YFP-FADD-DD expression plasmids as indicated then treated with the caspase inhibitor (zVAD.fmk, 0.1 mM) or serine protease inhibitor (AEBSF, 0.3 mM) as indicated. The percentage of injected cells that survive were determined after 24-h incubation, indicating that neither zVAD.fmk or AEBSF alone could prevent FADD-DD–induced cell death but the combination of both inhibitors did prevent death. Data shown are means ± SEM from five separate experiments.

tors like AEBSF but not by caspase inhibitors (Elliott *et al.*, 2000). To test whether a serine protease inhibitor could prevent FADD-DD–induced apoptosis, we injected normal prostate epithelial cells with FADD-DD expression plasmids then treated cells with zVAD.fmk or AEBSF and monitored cell survival by determining the fate of each FADD-DD–expressing cell.

Figure 4 shows that neither zVAD.fmk nor AEBSF could prevent cell death when added on their own. However, when we treated FADD-DD–expressing cells with both inhibitors simultaneously, they survived. Some of the control YFP cells underwent cell division, resulting in an apparent survival of >100%, whereas the FADD-DD–expressing cells did not rise above 100% even in the presence of both inhibitors. This may reflect a separate effect of the protease inhibitors or FADD-DD inhibiting cell growth. Because both the caspase inhibitor and serine protease inhibitor are required to prevent cell death, these data suggest that separate caspase and serine protease signals are activated by FADD-DD and that one enzyme is not upstream of the other. Furthermore, if one signal is blocked, the other protease is sufficient to kill the cells. Because it has been reported that zVAD.fmk can inhibit cathepsin B (Schotte *et al.*, 1999), we tested whether a cathepsin B–specific inhibitor (CA-074-ME) could cooperate with AEBSF to prevent FADD-DD–induced cell death. The cathepsin B inhibitor did not mimic the effect of zVAD.fmk (unpublished data) indicating that caspases themselves are important in the response.

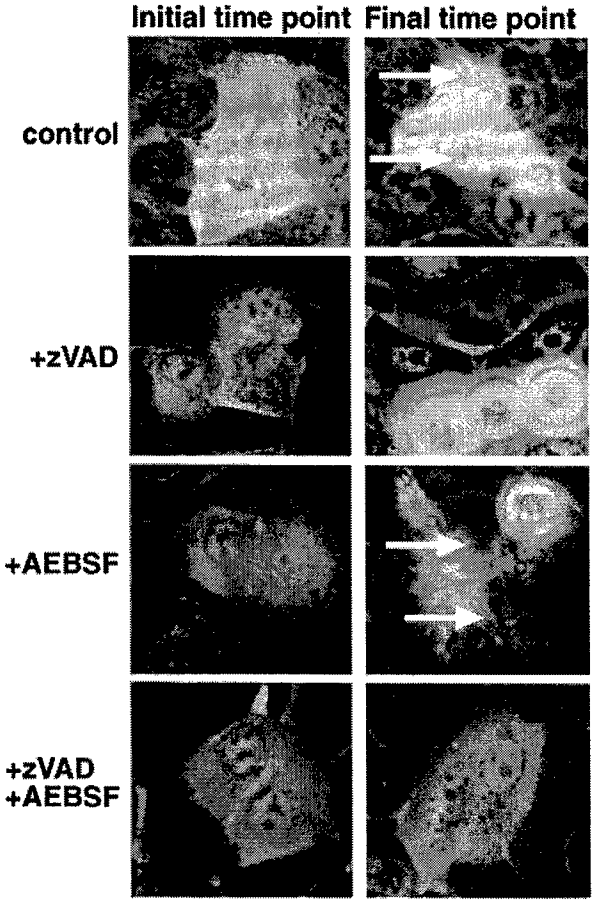

Figure 5. Caspases and serine proteases regulate different aspects of FADD-DD–induced apoptosis. Normal prostate cells were injected with the YFP-FADD-DD expression plasmid then incubated with zVAD.fmk or AEBSF as indicated. Time-lapse fluorescence microscopy was performed to monitor the response of each injected cell. The images show overlayed fluorescence and phase images of the same cells at the beginning and end of the experiment indicating that FADD-DD causes membrane blebbing and cell fragmentation that is blocked by zVAD.fmk but not by AEBSF. Quicktime movies of these experiments are contained in the supplementary material.

Caspases and Serine Proteases Have Different Effects on the Morphology of Dying Cells

There are examples in the literature where other proteases and caspases induce similar apoptotic phenotypes presumably by cleaving the same substrates (Foghsgaard *et al.*, 2001). There are also examples where the phenotypes of cells dying in response to stimuli that can activate both caspases and other death signals is quite different (McCarthy *et al.*, 1997). To determine whether normal prostate epithelial cells dying in response to FADD-DD utilize caspase- and serine protease-dependent signals to kill cells in different ways, we used time-lapse microscopy. Figure 5 shows frames from time-lapse series of FADD-DD–injected normal prostate cells in the absence or presence of zVAD.fmk and AEBSF. Fluorescence images are overlayed on the phase image. The figure shows the same cells at the initial time point and the

final time point (6–7 h for the control, zVAD.fmk, and AEBSF-treated cells and 15 h for the zVAD.fmk +AEBSF–treated cells). The green cells express FADD-DD. Quicktime movies of this experiment are included in the supplementary material. With no inhibitors, FADD-DD–expressing cells contract and display numerous membrane blebs (arrows) that retain the fluorescent protein as is typical of caspase-dependent apoptosis. In the presence of zVAD.fmk, the dying cells contract, round up, and detach from the dish but do not display membrane blebs. Conversely, FADD-DD–injected cells dying in the presence of AEBSF with no zVAD.fmk display typical hallmarks of caspase activity such as membrane blebs. In agreement with the cell survival data shown in Figure 4, most of the cells that express FADD-DD in the presence of both zVAD.fmk and AEBSF remain flat and attached to the dish. Thus, the caspases that are activated by FADD-DD in normal epithelial cells cause the distinct membrane blebbing that is characteristic of the dying cells.

Bcl-xL Inhibits Caspase-dependent Phenotypes in FADD-DD–induced Apoptosis

Bcl-xL blocks release of cytochrome c and other mitochondrial proteins. We examined the morphology of cells that coexpressed FADD-DD and Bcl-xL. Bcl-xL prevented membrane blebbing but did not prevent cell rounding (Figure 6A). This suggests that Bcl-xL inhibited caspase activation but not the serine protease that is inhibited by AEBSF. Similar inhibition of blebbing was observed when we coexpressed FADD-DD with a dominant-negative version of caspase 9 (dn9), which has a cysteine-serine mutation at the active site. These data suggest that FADD-DD activates caspase 9 through the mitochondrial pathway and Bcl-xL should inhibit FADD-DD–induced caspases. We tested this hypothesis by directly measuring caspase activity in cells that were injected with FADD-DD using a FRET-based method (Morgan and Thorburn, 2001). The method allows the continual measurement of caspase activity in individual cells. We monitored changes in caspase activity for 120 min before the beginning of cell contraction and rounding in FADD-DD–expressing cells in the presence or absence of Bcl-xL. This is achieved by monitoring changes in the ratio of yellow (FRET) fluorescence/cyan fluorescence emitted from a CFP-YFP fusion that can be cleaved by caspase 3 and other effector caspases. Figure 6B shows caspase activity in seven FADD-DD– and seven FADD-DD+Bcl-xL–expressing cells. FADD-DD causes activation of a caspase that can cleave the FRET probe. This is shown by a rise in caspase activity as measured by a loss of FRET and increase in cyan fluorescence resulting in a change in the yellow/cyan ratio.

The increase in caspase activity begins abruptly 30–60 min before each cell began to contract, which was designated as time 0. Bcl-xL inhibited this caspase activation and there was no detectable increase in caspase activity in any of the Bcl-xL–expressing cells. Note, however, that like the FADD-DD alone cells, each of the cells expressing Bcl-xL did undergo cell contraction and rounding, beginning at the time point designated as 0 min. Caspases presumably become active shortly after the release of cytochrome c, which Green and colleagues have demonstrated to be rapid and coordinated within each cell (Goldstein *et al.*, 2000). Together, these data suggest that the caspase activation occurs

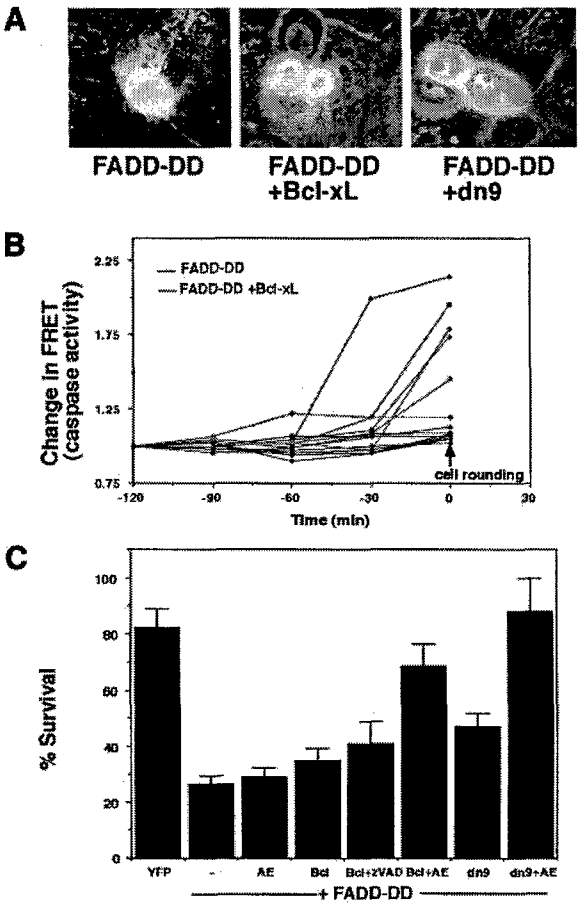

Figure 6. Bcl-xL inhibits activation of caspases by FADD-DD. FADD-DD–expressing cells were coinjected with control, Bcl-xL, and dominant-negative caspase 9 (dn9) expression plasmids. Panel A shows the morphology of injected cells. Bcl-xL and dn9 inhibit the membrane blebbing that is typically observed with FADD-DD alone. The images are overlayed fluorescence and phase images, green cells express the YFP-tagged FADD-DD molecule. Panel B shows caspase activation in seven cells injected with FADD-DD (red lines) and seven cells injected with FADD-DD plus Bcl-xL (blue lines) along with the expression plasmid encoding the cyan-yellow FRET probe. Fluorescence images were captured for yellow and cyan fluorescence, and the ratio of yellow/cyan fluorescence per unit area was calculated for each time point. The inverse change in FRET ratio indicative of caspase activation was plotted after normalization for each cell. Quantitation was for 120 min before cell contraction and rounding began (designated time 0). FADD-DD causes an abrupt increase in caspase activity that is prevented by Bcl-xL. Panel C shows cell survival of FADD-DD injected cells, cells injected with FADD-DD plus Bcl-xL or cells injected with FADD-DD plus dominant-negative caspase 9 (dn9) in the presence or absence of AEBSF (AE) or zVAD.fmk (zVAD) where indicated. FADD-DD induces cell death that cannot be blocked by AEBSF, zVAD, Bcl-xL, or dn9 alone. Bcl-xL and zVAD.fmk do not cooperate to prevent FADD-DD–induced death. Both Bcl-xL and dominant-negative caspase 9 cooperate with AEBSF to inhibit FADD-DD–induced cell death, resulting in cell survival that is similar to the YFP control. Data are means ± SEM from between 4 and 12 experiments for each sample.

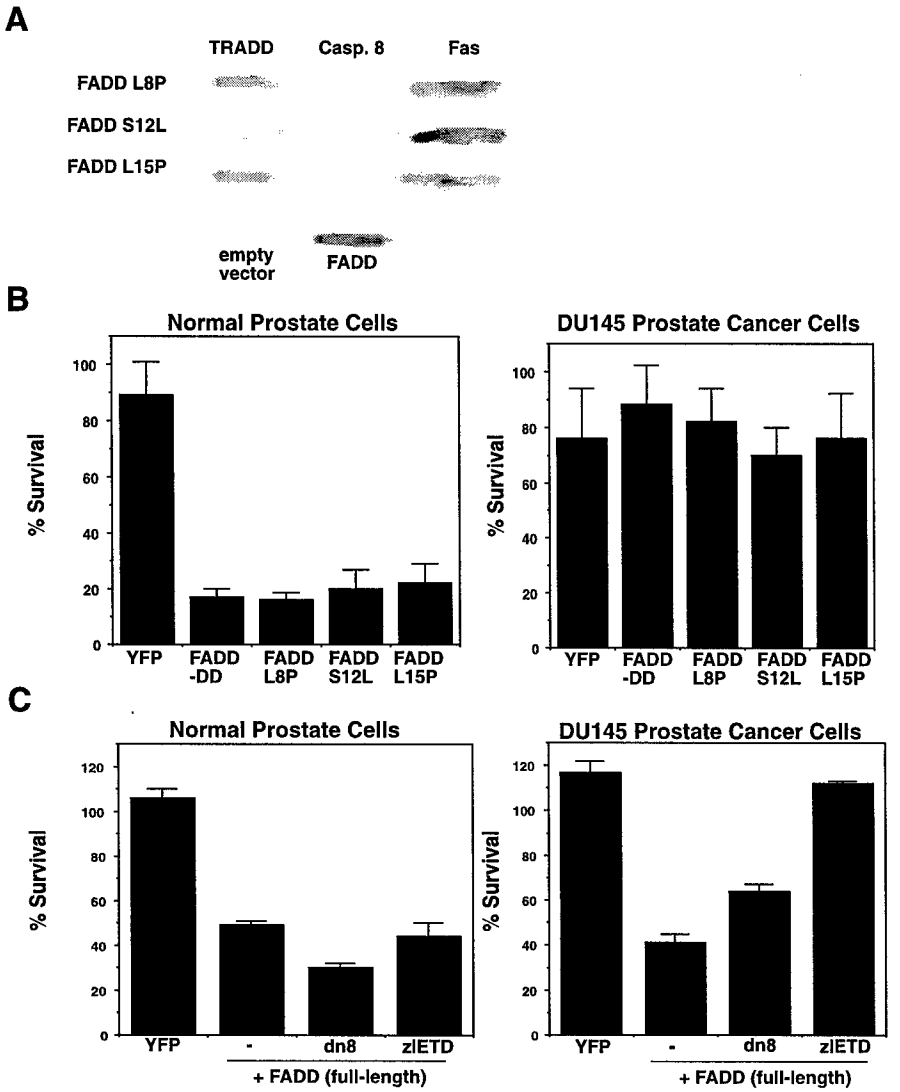

Figure 7. Full-length FADD kills normal prostate cells via a caspase 8–independent mechanism. (A) Yeast β-galactosidase filter assays of directed two-hybrid interactions of full length FADD mutants that were selected by reverse two-hybrid screening for failure to interact with caspase 8 while retaining the ability to interact with Fas. FADD L8P and L15P mutants bound both TRADD and Fas but not caspase 8. The S12L mutant did not bind caspase 8 or TRADD. Controls show β-Gal assays of yeast expressing the empty vector and wild-type FADD binding to caspase 8. (B) Cell survival assays after microinjection of YFP, FADD-DD, or the L8P, S12L, and L15P full-length FADD mutants into normal prostate cells and DU145 prostate cancer cells. FADD-DD and the three full-length FADD mutants killed normal cells but not DU145 cells. (C) Cell survival after microinjection of YFP or wild-type FADD into normal prostate cells or DU145 cells. Wild-type FADD killed both normal cells and cancer cells. Coexpression of dominant-negative caspase 8 (dn8) or pretreatment with a caspase 8 inhibitor (zIETD) inhibited FADD-induced death in DU145 cells but not in normal prostate cells. These data indicate that FADD can kill normal prostate cells in a caspase 8–independent mechanism that is not active in DU145 cells.

through the mitochondrial pathway and that the serine protease that is inhibited by AEBSF is activated through a different mechanism. If this is correct, Bcl-xL or dominant-negative caspase 9 should both fail to inhibit FADD-DD–induced cell death on their own but instead should cooperate with AEBSF to prevent cell death. Conversely, Bcl-xL should not cooperate with zVAD.fmk to inhibit FADD-DD–induced death. Figure 6C shows that this is indeed the case. Cell death was efficiently blocked only by the combination of AEBSF with either Bcl-xL or dominant-negative caspase 9.

Full-length FADD Induces Apoptosis via a Caspase 8–independent Mechanism in Normal but not Cancerous Prostate Cells

The previous experiments were performed using the truncated FADD-DD molecule. Expression of full-length wild-type FADD induces apoptosis in all cells by virtue of its ability to activate caspase 8. To test if the same pathway could be activated by full-length FADD rather than just the truncated molecule, we first identified FADD point mutants that are unable to bind caspase 8 using a reverse two-hybrid screen (Thomas *et al.*, 2002). Reverse two-hybrid screens identify mutants that have lost the ability to interact with a particular protein. Our modified method requires that these mutants retain the ability to interact with a different protein and thus selects for mutants that lose specific binding interactions without affecting overall protein structure or stability. We screened >500,000 random FADD mutants and identified mutants that retained the ability to interact with Fas but could not interact with caspase 8. All the mutations were in the death effector domain of the protein. Three mutants (L8P, S12L, and L15P) were chosen for further analysis. All three mutants bound to Fas but not caspase 8. The L8P and L15P mutants also displayed wild-type binding to TRADD (Figure 7A). The mutants were made as GFP fusions and injected into normal prostate cells or DU145 tumor cells. If

the FADD-dependent apoptosis in normal cells is distinct from FADD's established mechanism of action through caspase 8, these mutants should behave like FADD-DD and kill normal prostate cells but not prostate tumor cells. Figure 7B shows that this was the case.

Although the FADD point mutants and the isolated FADD death domain are only able to kill normal epithelial cells, the wild-type protein can induce apoptosis in both normal and cancerous cells. We next tested if this death was inhibited by caspase 8 inhibitors. Coexpression of full-length, wild-type GFP-FADD with dominant-negative caspase 8 or treatment with a selective caspase 8 inhibitor (zIETD.fmk) inhibited apoptosis of prostate cancer cells but did not inhibit apoptosis of normal prostate cells (Figure 7C). These data indicate that FADD can activate two apoptosis pathways in prostate epithelial cells. The first pathway involves caspase 8 recruitment through the death effector domain and functions in both prostate cancer cells and normal prostate cells. The second pathway works through the FADD death domain, does not involve caspase 8 and only functions in normal cells.

TRAIL-induced Apoptosis of Normal Prostate Cells Occurs through Caspase- and Serine Protease-dependent Pathways

The previous experiments were performed using exogenously expressed FADD molecules. However, there is no reason to think that FADD signaling in response to physiological signals is mediated through regulation of FADD expression levels. Rather, FADD is activated by death receptors such as Fas, TNFR1, or the TRAIL receptors, and overexpressed FADD mimics the effects that occur in response to receptor signaling, e.g., by activating caspase 8. In most cases, apoptosis after activation of these receptors is inhibited by caspase inhibitors such as zVAD.fmk. There are, however, examples where these receptors induce caspase-independent apoptosis (Foghsgaard *et al.*, 2001) and necrosis (Vercammen *et al.*, 1998a, 1998b; Denecker *et al.*, 2001).

If death receptors activate the FADD-DD–dependent apoptosis pathway, we should find that normal prostate cell apoptosis induced by the relevant ligand would not be completely inhibited by zVAD.fmk alone but would show increased inhibition by zVAD.fmk plus AEBSF. Conversely, prostate tumor cells should be unable to activate the caspase 8–independent pathway and should therefore be maximally protected by zVAD.fmk alone. TNF-α did not efficiently kill normal prostate cells (unpublished data) but both Fas ligand– and TRAIL-induced apoptosis of normal prostate cells. TRAIL has been reported to be unable to kill normal human cells, including normal prostate epithelial cells (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999). However, other investigators have found that TRAIL can induce apoptosis of normal prostate epithelial cells (Nesterov *et al.*, 2002), thus supporting our observations.

TRAIL-induced death of normal prostate cells was only partially inhibited by zVAD.fmk as shown by the presence of many rounded and detached cells but was blocked by zVAD.fmk plus AEBSF (Figure 8). AEBSF alone did not prevent TRAIL-induced cell death. The caspase inhibitor did prevent membrane blebbing because TRAIL treatment in the presence of zVAD.fmk resulted in many rounded cells without noticeable blebs. TRAIL alone and TRAIL plus AEBSF

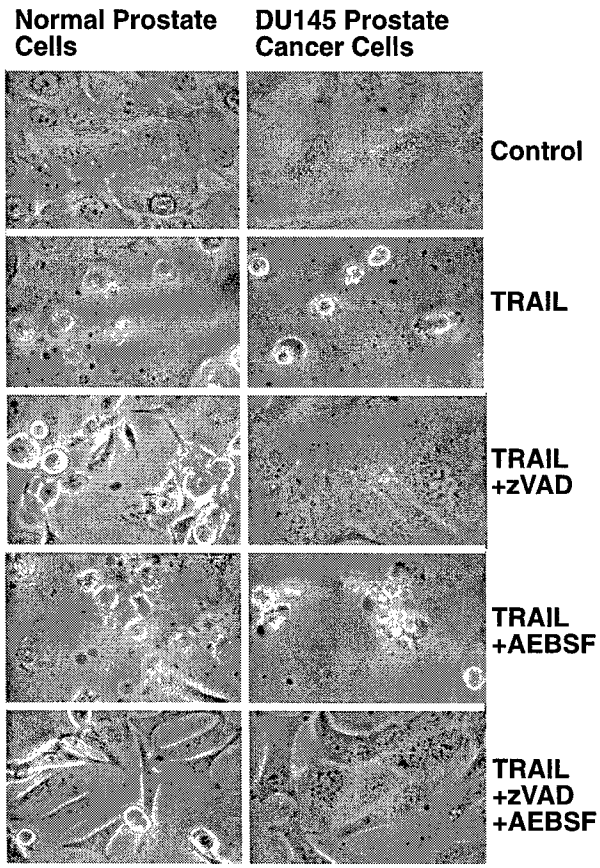

Figure 8. TRAIL induces apoptosis of normal but not cancerous prostate cells through an AEBSF-sensitive activity. Normal prostate epithelial cells and DU145 prostate cancer cells were treated with cycloheximide alone (control) or cycloheximide plus recombinant TRAIL in the presence of zVAD.fmk or AEBSF as indicated. Cell survival was determined by microscopy after 24 h. TRAIL killed both normal and cancerous prostate cells. The caspase inhibitor zVAD.fmk efficiently prevented cell death in the DU145 cells but did not prevent death in normal prostate cells. The addition of AEBSF and zVAD.fmk did prevent death of normal cells. These data indicate that TRAIL activates cell death pathways in normal prostate cells that have the same requirement for both caspase- and serine protease-dependent signals as FADD-DD.

treatments resulted in many cells with noticeable membrane blebs. The involvement of an activity that is inhibited by AEBSF was specific to the normal cells because TRAIL-induced death of DU145 cells was inhibited completely by zVAD.fmk. The addition of AEBSF did not confer added protection to DU145 cells. These data suggest that TRAIL can activate the conventional, caspase 8–dependent apoptosis pathway and the pathway that involves both caspase and AEBSF-sensitive signals in normal prostate cells but can only activate the caspase 8 pathway in cancer cells.

DISCUSSION

In this article, we show that the isolated death domain of FADD, which inhibits death receptor–induced apoptosis in

prostate tumor cells (Figure 2), can induce apoptosis in normal prostate cells. This response occurs only in normal epithelial cells, whereas epithelial cancer cells are resistant. Normal prostate fibroblasts and smooth muscle cells do not undergo FADD-DD–induced apoptosis (Morgan *et al.*, 2001). These data, along with our previous identification of point mutants that do not induce apoptosis (Morgan *et al.*, 2001), indicate that FADD-DD–induced apoptosis is not a nonspecific event. Rather, we suggest that the FADD death domain can activate a cell type–specific apoptotic pathway that functions in normal epithelial cells but is defective in tumor cells. Expression of exogenous wild-type FADD can activate caspase 8 to induce apoptosis of both normal cells and cancer cells. The apoptosis that occurs only in normal cells was therefore only apparent when we used a truncated protein that contains just the death domain, full-length FADD point mutants that cannot bind caspase 8 or when we inhibited caspase 8 in other ways.

FADD-DD activates the mitochondrial caspase-activation pathway by a Bcl-xL–sensitive mechanism to stimulate caspase 9 and then 7, 6, and 3 in normal prostate cells. In addition, at least one serine protease that can be inhibited by AEBSF is activated in normal cells by FADD-DD. There are several examples where noncaspase proteases are upstream of caspases. This can be achieved by digestion of the same signaling proteins (e.g., Bid) that are targeted by initiator caspases (Pinkoski *et al.*, 2001; Stoka *et al.*, 2001). In this case, an inhibitor of the upstream protease should prevent caspase activation. There are also examples where other proteases appear to be downstream of caspases (Jones *et al.*, 1998; van Eijk and de Groot, 1999; Foghsgaard *et al.*, 2001). In this case, a caspase inhibitor should prevent activation of the downstream protease. Activation of noncaspase proteases and caspases may also be mechanistically unrelated, and one class of protease may not be required for the activation of the other. FADD-DD activation of caspases and the AEBSF-sensitive serine protease in normal prostate cells is an example of the latter situation, because caspase and serine protease inhibitors must be combined to prevent cell death (Figure 4). Moreover, the morphology of the dying cells is different when caspase inhibitors or serine protease inhibitors are used, suggesting that the two types of protease target different substrates. If caspases are blocked, the dying cells do not display membrane blebbing and cell fragmentation but do round up and detach from the dish. Conversely, membrane blebbing is active in FADD-DD–expressing cells that are treated with the serine protease inhibitor (Figure 5). The requirement for caspase activity for membrane blebbing is well established and can be achieved by caspase cleavage of ROCK1 (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001). These data suggest the model shown in Figure 9.

There are other proteins that activate both caspase and serine protease-dependent pathways to kill cells. For example, the mitochondrial serine protease, Omi/HtrA2, is released into the cytoplasm where it can bind to and inhibit Inhibitor of Apoptosis proteins (Suzuki *et al.*, 2001; Hegde *et al.*, 2002; Martins *et al.*, 2002; van Loo *et al.*, 2002; Verhagen *et al.*, 2002). This leads to increased caspase activity. In addition, Omi/HtrA2's serine protease activity can induce an atypical form of apoptosis (Suzuki *et al.*, 2001; Verhagen *et al.*, 2002). Although it is attractive to suggest that activation of Omi/HtrA2 might be responsible for all the effects that

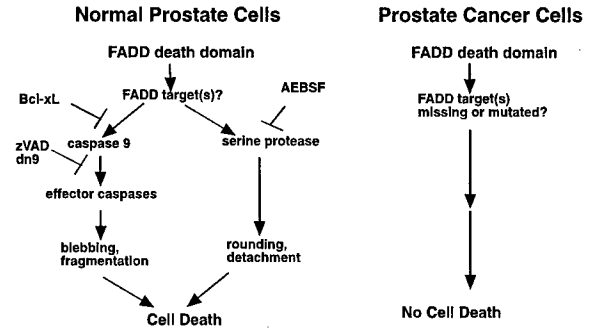

Figure 9. Model of FADD-DD–induced apoptosis signaling in normal prostate epithelial cells. FADD-DD works through unidentified effectors to activate caspase 9 through a mitochondrial caspase activation pathway that is Bcl-xL sensitive. This leads to the activation of effector caspases, which cause cell death that is associated with membrane blebbing and cellular fragmentation. In addition, a serine protease pathway is activated that can be inhibited by AEBSF but not by Bcl-xL, which causes cell death that is associated with cell rounding and detachment. This pathway is not activated in prostate cancer cells because these cells possess defects in effector molecules that mediate the death domain signal.

we observe with FADD-DD, we think this is unlikely for two reasons. First, caspase-independent death by Omi/HtrA2 has been reported to occur only when the serine protease is highly expressed (Martins *et al.*, 2002). This suggests that physiological levels of this enzyme such as would be released in our cells may not kill by a serine protease-dependent mechanism. Second, Bcl-xL, which would presumably block the release of Omi/HtrA2, did not prevent FADD-DD–induced death but instead only prevented caspase activation and the caspase-dependent morphological phenotypes (Figure 6). This suggests that release of mitochondrial proteins is responsible for caspase activation but not for activation of the serine protease.

We previously found that the ability of FADD-DD mutants to interact with Fas or TRADD did not completely correlate with the mutants' ability to induce normal prostate cell apoptosis (Morgan *et al.*, 2001). This implies that the truncated FADD is not functioning as an inhibitor of, for example, a Fas-induced survival signal. Instead, our data are more consistent with an active death pathway that is stimulated by the FADD death domain. TRAIL-induced death in normal prostate epithelial cells shows the same requirement for zVAD.fmk- and AEBSF-sensitive signals as FADD-DD. Therefore, TRAIL receptors may stimulate this FADD-dependent pathway under normal circumstances. We are further analyzing TRAIL- and FADD-DD–induced apoptosis in normal and cancerous prostate cells to test this hypothesis.

Apoptosis is a primary defense against cancer development (Hanahan and Weinberg, 2000; Green and Evan, 2002); however, apoptosis signaling pathways that perform this function have not been well characterized. Apoptosis pathways that serve to protect against cancer development should function in normal cells but not in cancer cells and may be cell type specific. Because FADD-DD has these characteristics, we suggest that the signaling pathway that is activated by FADD's death domain (perhaps in response to TRAIL) in normal epithelia suppresses carcinoma develop-

ment. Further analysis of the mechanism of FADD-DD–induced apoptosis in normal epithelial cells and the mechanism of resistance in cancer cells may therefore provide new insights into the development of epithelial cancers and could identify new targets for cancer therapeutics. To this end, we are using genetically defined human epithelial cells to determine at which stage during the immortalization and transformation process epithelial cancer cells become resistant to FADD-DD–induced apoptosis.

ACKNOWLEDGMENTS

We thank David Virshup for useful discussions and comments on the manuscript. We are grateful to Guy Salveson for providing the dominant-negative caspase 8 and 9 cDNAs. This work was supported by grants to A.T. from the Department of Defense, North Carolina Biotechnology Center and Wake Forest University. M.J.M. was supported by an National Cancer Institute training grant in Signal Transduction.

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Review article

Death receptor-induced cell killing

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Received 6 July 2003; accepted 15 August 2003

Abstract

Apoptosis pathways activated by death receptors of the tumour necrosis factor (TNF) family such as Fas, TNFR1, or the TRAIL receptors DR4 and DR5 are implicated in diverse diseases. These are also the best-understood apoptosis pathways and many of our ideas about apoptosis regulation come from studying these pathways. Cell killing from such receptors occurs because of recruitment to the receptor of the adaptor protein FADD, which in turn recruits the pro form of caspase-8. Aggregation of pro-caspase-8 leads to its auto-activation and subsequent activation of effector caspases such as caspase-3. The apoptotic signal can be amplified through the mitochondria and inhibited through the action of competing molecules such as the inhibitor c-FLIP, which binds to the receptor complex in place of caspase-8. This simple mechanism explains much of the cell death that is induced by death receptors. However, recent studies indicate that we must incorporate new information into this model. Some examples that add new layers of complexity will be discussed in this review.

Keywords: Death receptors; TNF; Caspase-8

1. Introduction

Two main pathways that activate caspases, the proteases that are the primary drivers of apoptosis, have been identified. Diverse stress pathways cause release of mitochondrial proteins to activate the "intrinsic pathway" [1]. Mitochondrial protein release often occurs after BH-3-only members of the Bcl-2 family [2,3] bind to and neutralize anti-apoptotic members of the Bcl-2 family. This promotes release of mitochondrial proteins including cytochrome *c* through an as yet incompletely characterized mechanism. Released cytochrome *c* interacts with Apaf-1, pro-caspase 9 and dATP to form a complex called the apoptosome [4]. This complex dimerizes and activates caspase 9, which then activates effector caspases to induce apoptosis. Other released mitochondrial proteins that promote apoptosis include Apoptosis Inducing Factor (AIF) [5], Smac/Diablo [6,7], Endonuclease G [8] and Omi/HtrA2 [9–12]. Recent work suggests that these mitochondrial effects might occur downstream of other caspases, particularly caspase-2 [13–15] and that Bcl-2 proteins might function at this stage too, i.e. before any mitochondrial activity [3,15].

The other apoptotic pathway, the "extrinsic pathway", which is activated by ligand-bound death receptors such as tumour necrosis factor (TNF), Fas or TRAIL receptors, has been thought to be much simpler and better understood [16]. Although death receptors can promote cell growth under at least some situations [17], the ability of these receptors to induce apoptosis is critical in several disease processes and has been the focus of most work to date. The six known death receptors contain an intracellular globular protein interaction domain called a death domain (DD). Upon ligand binding to death receptors probably in the form of pre-associated receptor trimers [18,19], activated death receptors recruit an adaptor protein called Fas Associated Death Domain (FADD) [20]. FADD consists of two protein interaction domains: a death domain and a death effector domain (DED). The current model is that FADD binds (directly or via another adaptor such as TRADD, which binds to TNFR1) to the receptor through interactions between DDs and to pro-caspase-8 through DED interactions to form a complex at the receptor called the Death Inducing Signalling Complex (DISC). Recruitment of caspase-8 through FADD leads to its auto-cleavage and activation [21]. Active caspase-8 in turn activates effector caspases such as caspase-3 causing the cell to undergo apoptosis by digesting upwards of a hundred or so proteins [22]. An endogenous inhibitor, c-FLIP, which is related to caspase-

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doi:10.1016/j.cellsig.2003.08.007

8 but has no protease activity, is thought to function by competing with caspase-8 for binding to the DISC [23,24]. The BH3-only protein Bid is cleaved by caspase-8 and is then translocated to the mitochondria to activate the intrinsic pathway [25], thus connecting the two caspase activation pathways and amplifying the death receptor apoptotic signal. Thus, the current view of the mechanism of death receptor-induced apoptosis involves the formation of protein complexes that lead to activation of caspases and amplification of the death signal through the mitochondrial apoptosis pathway. Some recent findings that add new levels of complexity to this model will be discussed in this review.

2. Do death domains and death effector domains bind independently to their targets?

The current model is that FADD interacts with the receptor (e.g. in the case of Fas) or another adaptor (e.g. TRADD in the case of TNFR1) through its DD. At the same time, the other interaction domain in FADD, the DED, interacts with a DED on caspase-8. This model implies that the DD and DED function independently of each other and that these domains and the related CARD domain, which have similar structures made up of six alpha helices [26], homodimerize but do not heterodimerize. That is, a DD will interact with another DD and a DED will interact with another DED but the DED is not involved in the DD interaction and vice versa. Evidence for this idea comes from the fact that the isolated FADD DD can bind to Fas or TRADD. Indeed, this is the mechanism by which the isolated FADD DD functions as a dominant negative inhibitor of Fas-, TNFα- or TRAIL-induced apoptosis [27]. Solution structures of the isolated FADD and Fas DDs support this model and suggest that interaction takes place on a charged surface patch [28,29]. Targeted mutagenesis experiments further suggest that the same surface is involved in the FADD–TRADD interaction [30]. It was therefore surprising when a random genetic screen to identify point mutants in the FADD cDNA that prevent binding to Fas identified a series of mutations that affected the DED, not the DD [31]. The experiment used a modified method of reverse two-hybrid screening to identify point mutants in FADD that cannot interact with the Fas DD. As the name suggests, reverse two hybrids screen for loss of protein–protein interactions. A library of random mutants is generated and those that cannot interact with a target protein identified. This approach often identifies uninformative mutants that have severely disrupted protein structure or altered protein stability. In the modified method, such mutants are avoided by simultaneously screening for retention of interaction with a different protein (in this case TRADD).

According to the current model, a screen for FADD mutants that cannot interact with the DD of Fas but can interact with the DD of TRADD should identify mutations in the DD of FADD. Some mutants in the FADD DD with these characteristics have been identified [31] (L. Thomas and A.T., unpublished observations). However, the majority of the mutants that were identified in the reverse two-hybrid screen for FADD mutants that cannot interact with Fas were in the FADD DED. Specifically, mutations of amino acids in the loops that flank helix five of the DED abolished interaction with Fas but had no effect on the TRADD–FADD interaction. When these mutants were expressed in FADD-deficient cells, they rescued TNF-induced signalling (which requires TRADD binding) but could not rescue Fas ligand-induced signalling. This suggests that the binding specificity in mammalian cells is the same as in yeast. The simplest interpretation of these data is that both the DED and DD of FADD participate in the interaction with death receptors such as Fas and that the current model whereby different domains (i.e. DDs, DEDs and CARD domains) function independently of each other are oversimplified. A direct test of this idea will require structural information of the intact proteins rather than the isolated domains that have been used for previous structural studies. Because the mutations in the DED affect Fas but not TRADD binding, these data suggest that specificity of interactions between FADD and death receptors can be achieved through regulating the structure of the DED. This represents a new mechanism by which regulation could be imposed on these systems and could be used by cellular proteins to allow regulated activation of FADD by some receptors but not others. In addition, practical benefits might be obtained based on these ideas. For example, it may be possible to identify small molecules that alter the DED structure in a way that would inhibit Fas-induced apoptosis without affecting TNFR1 signalling. In recent work, we (L. Thomas et.al., manuscript in preparation) have identified a similar role for the FADD DED in the recruitment to the activated TRAIL receptors DR4 and DR5 indicating that other receptors also regulate DISC formation through the FADD DED.

3. How are caspases activated by death receptors?

The above discussion suggests that the binding of the adaptor that brings pro-caspase-8 to the activated death receptor complex is more complicated than previously thought. Other studies suggest that the next step is also not quite as simple as we previously thought. It was initially thought that pro-caspase-8 was brought to the receptor complex by FADD and that this resulted in self-activation of the caspase through a cross-proteolysis mechanism. That is, two pro-caspase-8 molecules were brought in close proximity and one molecule could digest the other molecule resulting in the formation of active, processed caspase-8. However, recent data indicate that caspase-8 activation occurs as a result of dimerization rather than processing per se and that the initial processing steps arise through intramolecular digestion rather than intermolecular digestion. Support for this model comes from experiments showing that regulated dimerization of a wildtype procaspase-

8 molecule and a mutant that does not have a functional active site can induce catalytic activity [32].

Interestingly, naturally occurring molecules that are effectively catalytically inactive pro-caspase-8 molecules are important regulators of death receptor-induced apoptosis. These are the cellular FLICE-like Inhibitory Protein (c-FLIP) proteins [24,33]. c-FLIP comes in two main isoforms, cFLIP-long and c-FLIP-short. Both proteins possess two DED motifs that are very similar to the DED's on pro-caspase-8. The short c-FLIP isoform consists of only these DED domains. However, the long protein also has a domain that is homologous to the catalytic domain of caspase-8. The long form of c-FLIP is not an active protease because it has several alterations that affect the active site of the enzyme. Both c-FLIP proteins and several viral proteins that are homologous to FLIP-short were originally thought to be solely inhibitors of death receptor-induced apoptosis that function by competing with pro-caspase-8 for binding to FADD at the activated DISC. In support of this view, increased levels of c-FLIP are associated with cancer and can confer protection against Fas ligand- or TRAIL-induced apoptosis [24,33]. However, recent work shows that the long form of FLIP when recruited to the DISC can actually promote caspase-8 activation [34,35]. This occurs because the FLIP-L and procaspase-8 dimerize and this activates the proteolytic activity of the caspase-8 molecule. One conclusion from this work is that caspase-8 activation can occur even in the absence of caspase-8 processing, thus supporting the conclusions arising from studies of artificially dimerized caspase-8 molecules. These data suggest that FLIP-L and FLIP-S are not equal in their ability to inhibit death receptor-induced apoptosis and indeed FLIP-L may promote apoptosis at least when expressed at certain levels. These findings could have important consequences for understanding cellular responses to various signalling events. For example, protein synthesis inhibitors can promote apoptosis induced by death receptors because they reduce FLIP levels [36]. Subtle differences in the relative levels of FLIP-L, FLIP-S and procaspase-8 might activate or inhibit caspase activity depending on the particular stoichiometry of these molecules at the DISC.

Detailed analysis of the mechanism of activation of the wildtype procaspase-8 protein (i.e. without artificial dimerization domains) supports the above studies. Using biochemical and biophysical approaches, it was demonstrated that dimerization is required and sufficient for caspase-8 activation but that caspase-8 processing is neither necessary nor sufficient for catalytic activity [37,38]. Rather than being required for activity, cleavage appears merely to stabilize the caspase-8 dimer. The fully processed, stable caspase-8 dimer may also have a different substrate specificity in vivo compared with the unprocessed or partly processed, active caspase-8 dimer. The idea is that active, dimerized but incompletely processed caspase-8 may remain bound to FADD at the DISC. Such a caspase-8 molecule could only digest potential substrates that are also localized in the proximity of the DISC. Important caspase-8 substrates are indeed found at the DISC including the protein kinase RIP [39] and components of the actin cytoskeleton, which must be digested by caspase-8 to promote Death Receptor internalization [40]. However, many other important caspase-8 substrates including pro-caspase-3 and Bid are less likely to be in close proximity to the DISC. Therefore while dimerization causes activation of caspase-8, this in itself may not be sufficient to allow all potential caspase-8 substrates in the cell to be digested. Because a procaspase-8-FLIP-L dimer can be catalytically active but is not completely processed, this molecule might also remain anchored at the DISC and thus be available to digest substrates that are also at this site.

An important practical consequence of these findings is that identification of the processed forms of caspase-8, which has been widely used as an assay for caspase-8 activation, is probably no longer valid. Instead, it should be borne in mind that the presence of non-processed but activated caspase-8 may lead to the cleavage of a subset of potential substrates, which could in turn stimulate only some of the cellular responses associated with apoptosis. It will be interesting to determine if different substrates are indeed cleaved in vivo by partially processed, compared with fully processed caspase-8 and to determine how this affects cellular responses.

4. Do these interactions always occur at the cell membrane?

Fas and TRAIL receptor DISC immunoprecipitations show that a stable ligand–receptor complex that contains FADD and caspase-8 occurs after receptor activation and it is therefore thought that the initial caspase activation events that lead to receptor-induced cell death occur at the cell membrane. However, important pro-apoptotic signalling events involving these proteins may also occur at other locations in the cell. In the case of the TNFR1 apoptotic signal, it was recently shown that while TRADD, RIP and TRAF2 were bound to the receptor, FADD and caspase-8 were not detectable in the complex. Nevertheless, the authors concluded that the apoptotic signal required FADD and caspase-8 and suggested that the activation of these molecules must occur somewhere other than at the membrane-bound receptor [41]. If this idea is correct, then we might expect to find TRADD, FADD, etc., at other locations in the cell. Recent studies found that this is in fact the case. TRADD was shown to have nuclear import and export sequences that cause rapid shuttling between the cytoplasm and the nucleus and inhibition of nuclear export with leptomycin B causes accumulation of TRADD in nuclear structures that are associated with promyelocytic leukemia protein (PML) nuclear bodies [42]. The nuclear accumulation of TRADD is tightly regulated but the precise details of this regulation and the physiological signals that cause nuclear accumulation are still unclear (M. Morgan and A.

Thorburn, unpublished data). Similarly, FADD has been reported to have nuclear import and export sequences and to be primarily localized in the nucleus [43,44]. It is not yet clear how the nuclear localization of these adaptors is related to their death receptor activities and it is possible that these unexpected localizations indicate a different function for these molecules. For example, nuclear FADD may be involved in the response to DNA damage because nuclear FADD is associated with the methyl–CpG binding domain protein 4, which is involved in the mismatch repair apparatus [43]. Similarly, apoptosis by an exclusively nuclear TRADD truncation mutant could be distinguished from caspase-8-dependent apoptosis (i.e. the kind of apoptosis that we expect to be induced by activated death receptors) [42], further suggesting a role that is distinct from death receptor signalling. Indirect evidence for signalling from intracellular compartments by components of the death receptor complexes comes from studies with LMP1, a viral protein that mimics some death receptor signalling pathways. This molecule uses many of the same signalling proteins as the death receptors but while it can be localized at the cell membrane, the signalling occurs from intracellular compartments [45].

While we cannot exclude the possibility that the nuclear and other intracellular signalling by these proteins may be important mainly in response to stimuli other than death ligands, these findings lend support to the view that there are important functions for these proteins at locations other than the cell membrane. By determining where within the cell the initial caspase activation events in response to death ligands occur, using Fluorescence Resonance Energy Transfer to measure caspase activity [46–50], it should be possible to determine how these activities fit into the death receptor signalling pathways.

5. How many ligand-bound receptors are needed to induce apoptosis?

Soluble death ligands such as Fas ligand (FasL) are not as potent as membrane-bound ligands. An explanation for this observation comes from the finding that a single FasL molecule bound to a Fas receptor trimer is not able to induce apoptosis. Interestingly, apoptosis (and other Fas-dependent signalling including JNK phosphorylation) can be restored if two soluble FasL molecules are physically linked [51]. Thus the active death receptor complex that is able to signal apoptosis probably consists of at least two ligands bound to a hexameric receptor consisting of two pre-formed trimers [18]. These data explain why soluble epitope-tagged versions of death ligands are significantly more pro-apoptotic if they are crosslinked by antibodies that recognize the tag. Why must we have two receptor trimers each bound by linked ligands in order to activate caspase-8? One simple explanation is that a single ligand-bound receptor trimer is unable to bind two procaspase-8 molecules and thus unable to promote caspase dimerization. Determination of the stoichiometry of the various components of the DISC (i.e. receptors, FADD, procaspase-8, etc.), combined with structural analysis of active and inactive DISCs, should provide an answer to this question. Differences in the number of receptors that must be activated to induce efficient death signalling may have important practical consequences for the development of therapeutics based on death receptor agonists and antagonists.

6. Caspase-independent death induced by death receptors

It was initially thought that all death receptor-induced cell killing was achieved through caspase activation and the above discussion has focused on how caspase-dependent cell death occurs. However, there are numerous reports of programmed cell death in response to activation of death receptors even when caspases are inhibited, suggesting that caspase-independent pathways can be stimulated by death receptors. Examples include death induced by Fas or TNFα that has been characterized as necrotic [52–54].

In at least some cases, the decision to undergo caspase-dependent apoptosis or caspase-independent necrosis may involve the heat shock protein HSP90, which may alter the components of the DISC to activate either apoptosis or necrosis [55]. Fas-induced necrosis has also been reported to require signalling through the DISC-interacting kinase RIP [56]. While this response (unlike some other RIP-dependent response such as NFκB signalling) requires the kinase activity, the relevant substrate is not known. There have been few studies that identify the effectors (i.e. proteases other than the caspases) that mediate death receptor-induced death when caspases are not involved. An exception is the identification of the lysosomal protease, cathepsin B, as a mediator of TNF-induced cell death in some cancer cell lines [57]. Importantly, this pathway was not activated in other tumour cell lines, indicating that different cell types may be more or less able to activate non-caspase dependent signalling even in response to the same receptor activation events. Unlike the case with caspase activation, we have little understanding of how alternate effectors like cathepsin B are activated by the receptors.

Recently, our laboratory found that TRAIL could kill normal prostate epithelial cells through pathways that involve both caspases and a serine protease that seem to work in parallel pathways [58]. Importantly, the serine protease-dependent arm was not involved in killing of prostate cancer cells. This last finding further emphasizes that while the caspase activation pathway involving FADD-dependent caspase-8 recruitment works (as far as we can tell) in the same way in many if not all cell types, the caspase-independent mechanisms that death receptors use to kill cells may be highly cell-type dependent. Moreover, as in the case of prostate epithelial cells

responding to TRAIL, there may also be important differences that depend upon disease status. It seems likely that such specificity could have important biological significance but we will need to understand how these pathways are activated and controlled before we can fully interpret these differences. Most, perhaps all, the death receptors also activate other signalling pathways, e.g. to lead to NFκB, JNK or ERK signalling. In most cases, these pathways are thought to mediate pro-survival or growth signals but under some circumstances they may also participate in death signalling. Again, this may be cell- and tissue-type specific.

7. Some important remaining questions

A simple model of death receptor-induced apoptosis involving homotypic interactions between death domains on trimerized receptors and FADD and death effector domains on FADD and caspase-8 leading to caspase-8 cleavage and activation has become widely accepted. This model seems to be broadly correct but, as outlined above, new layers of complexity and opportunities for regulation are being added to it. Several important aspects remain obscure and need to be addressed experimentally. Structural studies of full-length proteins rather than the isolated domains that have been studied to date will be necessary if we are to understand how different domains (e.g. the death domain and death effector domain of FADD) interact with each other and with their binding targets. Similarly, we are just beginning to understand the stoichiometry of the death receptor complexes that signal cell death and we are just now coming to realize that the particular arrangement (e.g. of FLIP and caspase-8 molecules) and the location within the cell where these interactions occur might do more than switch apoptotic signalling on and off but could perhaps provide more subtle regulation and the activation of different apoptotic mechanisms. It will be important to understand these aspects of death receptor signalling because they may represent important regulatory steps that could be useful for targeted interventions. An obvious gap in our knowledge concerns the mechanisms and functional significance of caspase-independent death signalling by these receptors. We know it happens and we know some of the players that may be involved in the response but we have a long way to go to understand caspase-independent death signals even at the incomplete level of our understanding of caspase signals. Fortunately, we now have many of the tools to tackle these problems, so rapid progress seems assured. No doubt this progress will involve more surprising findings.

Acknowledgements

I thank Steve Kridel for providing comments on the manuscript. Research in our laboratory is supported by grants from the National Institutes of Health, the US Army Breast Cancer and Prostate Cancer Research Programs and the Susan G. Komen Breast Cancer Foundation.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 31, Issue of July 30, pp. 32780–32785, 2004
Printed in U.S.A.

Direct Binding of Fas-associated Death Domain (FADD) to the Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor DR5 Is Regulated by the Death Effector Domain of FADD*

Received for publication, February 16, 2004, and in revised form, June 1, 2004
Published, JBC Papers in Press, June 1, 2004, DOI 10.1074/jbc.M401680200

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Members of the tumor necrosis factor superfamily of receptors induce apoptosis by recruiting adaptor molecules through death domain interactions. The central adaptor molecule for these receptors is the death domain-containing protein Fas-associated death domain (FADD). FADD binds a death domain on a receptor or additional adaptor and recruits caspases to the activated receptor. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signals apoptosis through two receptors, DR4 and DR5. Although there is much interest in TRAIL, the mechanism by which FADD is recruited to the TRAIL receptors is not clear. Using a reverse two-hybrid system we previously identified mutations in the death effector domain of FADD that prevented binding to Fas/CD95. Here we show that these mutations also prevent binding to DR5. FADD-deficient Jurkat cells stably expressing these FADD mutations did not transduce TRAIL or Fas/CD95 signaling. Second site compensating mutations that restore binding to and signaling through Fas/CD95 and DR5 were also in the death effector domain. We conclude that in contrast to current models where the death domain of FADD functions independently of the death effector domain, the death effector domain of FADD comes into direct contact with both TRAIL and Fas/CD95 receptors.

Members of the tumor necrosis factor (TNF)[1] superfamily of receptors induce a variety of cellular responses including apoptosis, cellular differentiation, and proliferation. A subfamily of these receptors contains a death domain (DD) that is essential for transducing the apoptotic signal. Fas/CD95 is the best characterized member of this family. Binding of Fas ligand (FasL) to a preformed Fas/CD95 trimer (1) results in dimerization of two trimers (2) and higher levels of oligomerization (3). These activated receptors signal the apoptotic response by recruiting FADD to the cytoplasmic DD of the receptor to form the death-inducing signaling complex (DISC). FADD consists of two distinct domains, a DD that binds to the DD of Fas/CD95 and a death effector domain (DED) that binds to DEDs on caspase-8 and caspase-10 (4). The DD and DED have similar structural folds consisting of six anti-parallel α-helices, and both form globular protein structures whose only known function is to interact with other proteins (5). Thus, it is thought that binding of ligand to Fas/CD95 results in the recruitment of FADD through DD interactions followed by caspases through DED interactions. The induced proximity of two or more initiator caspases in a complex with FADD and the receptor results in their dimerization and activation (6, 7). Proteolytic processing leads to a fully processed, active form of the caspase that can dissociate from the receptor complex. Once activated, these caspases can then cleave and activate effector caspases such as caspase-3 and other substrates to induce the characteristic phenotypes associated with apoptosis.

Activation of TNF receptor 1 requires an additional adaptor protein, TRADD. Binding of TNFα to TNF receptor 1 results in the recruitment of TRADD (8) followed by FADD (9, 10), again through DD interactions. Similar to the Fas/CD95 receptor, recruitment of FADD to the complex leads to the recruitment and activation of caspase-8. Of note, recent studies suggest that the TRADD/FADD/caspase-8 complex dissociates from the receptor during TNF-induced apoptosis, implying an additional layer of regulation (11, 12). Nonetheless, FADD is essential for TNF-induced caspase activation because FADD-deficient cells fail to undergo apoptosis when treated with TNFα and a dominant negative form of FADD that consists of only the DD can block TNF-induced death (13, 14).

There is much interest in TNF-related apoptosis inducing ligand (TRAIL) because of its reported ability to induce apoptosis in tumor cells without affecting normal cells (15, 16), suggesting that it may be useful for treating cancer. There are two "signaling" receptors DR4 and DR5, which, similar to Fas/CD95, contain death domains. In addition, there are two "decoy" receptors, DcR1 and DcR2, that have truncated or completely absent DDs (17), suggesting that the DD is essential for apoptotic signaling through TRAIL. FADD is necessary for signaling through DR4 and DR5 because dominant negative FADD blocks TRAIL signaling (14), and FADD-deficient Jurkat cells do not undergo TRAIL-induced death (18). FADD, caspases-8 and caspase-10 are recruited to the TRAIL DISC, but TRADD was not detectable in this complex (19, 20). These reports led to a model similar to that of Fas/CD95 in which FADD binds directly to both TRAIL receptors rather than through an adapter molecule (21).

Recently, we made the surprising discovery that specific

* This work was supported by grants from the Susan G. Komen Breast Cancer Foundation (to L. R. T.), the United States Army Breast Cancer Research Program (to L. R. T. and A. T.), and National Institutes of Health Grant GM61694 (to J. C. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DISC, death-inducing signaling complex; DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; TRADD, TNF receptor-associated death domain; FasL, Fas ligand; GFP, green fluorescent protein.

mutations in the FADD DED could prevent binding to Fas/CD95 (22). This suggests that the FADD DED contributes to the interaction with the Fas/CD95 receptor and that the DED and DD of FADD do not function independently as suggested in previous models. Here we show that mutations in the DED of FADD also prevent binding of FADD to DR5 and are unable to rescue TRAIL signaling in FADD-deficient cells. Compensating second site mutations that restore binding of such a FADD mutation to DR5 were identified in the DED of FADD and restore TRAIL-induced activation of caspases, further indicating a role for the DED in coordinating the FADD-DR5 interaction. Using a reverse two-hybrid approach we were able to identify only one mutation in the FADD DD that showed differential binding between Fas/CD95 and DR5. These data suggest that the same residues in both the DED and DD of FADD regulate binding of FADD to Fas/CD95 and DR5.

MATERIALS AND METHODS

Reagents—Antibodies and reagents were purchased from the following sources: caspase-8 and caspase-3 (Cell Signaling, Beverly, MA), anti-FADD (Transduction Labs, Lexington, KY), anti-GFP (Chemicon, Temecula, CA), anti-actin and anti-FLAG (Sigma), FasL (Upstate, Charlottesville, VA), recombinant human TRAIL and anti-His_6 antibody (R & D Systems, Minneapolis, MN).

Plasmids—pGB14 was made by cloning the ADH1 promoter, Gal4 DNA-binding domain, multiple cloning site, ADH1 terminator cassette from pGBKT7 into the KpnI and SacI sites of pRS314. DR5 (amino acids 209–412), Fas (amino acids 177–335), and full-length catalytically inactive caspase-8 were made by PCR on the corresponding cDNA (23) and by cloning the product into pGB14. Full-length TRADD was cloned into pBTM-116. pACT3 and pcDNA-Puro plasmids have been described previously (22). Full-length FADD and FADD-DD (amino acids 79–208) were cloned into pEGFP-C2 or pACT3. The cytoplasmic domains of DR5 (amino acids 209–412) or Fas (amino acids 177–335) were cloned into pEGFP-C2. Amino acids 1–208 of FADD or 272–469 of DR5 were cloned C-terminally to FLAG. A more complete description of plasmids, maps, and sequences are available upon request.

Cell Lines—HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Jurkat cells were maintained in RPMI 1640 with 10% fetal bovine serum. FADD-deficient Jurkat cells (24) stably expressing FADD mutations were made by electroporating pcDNA3.1-Puro constructs and selecting for stable transformants as previously described (22).

Immunoprecipitation—HeLa cells were transfected with 2 μg of pEGFP construct plus 2 μg of FLAG or FLAG-FADD using FuGENE 6 (Roche Applied Science) in a 10-cm plate. After 18 h the cells were lysed in Triton X-100 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1× protease inhibitors), and the soluble fraction was incubated with 30 μl of M2-agarose (Sigma) for 4 h a 4 °C. The beads were washed four times in Tris-buffered saline, and precipitated GFP fusions were detected by immunoblot.

DISC Immunoprecipitations—20×10^6 BJAB cells were treated with 1.0 μg/ml nonspecific mouse IgG (Sigma) or monoclonal antibody 631 (R & D Systems, Minneapolis, MN) cross-linked with an equal amount of anti-mouse Fc (Sigma) for 30 min. The precipitations were carried out using protein A/G-agarose beads essentially as described (19), and the bound proteins were detected by immunoblot.

Two-hybrid Assays—Strains Y190 (*MATa his3 ade2 trp1 leu2 gal4 gal80 cyh2, LYS2:Gal1-HIS3 URA3:Gal1-LacZ*), DY6877 (*MATa ade2 can1 his3 leu2 lys2 trp1 URA3:8xLexA-LacZ*), and LY26 (*MATα can1 his3 leu2 met15 trp1 ura3 gal4::hisG gal80::hisG LYS2::LexA(op)-HIS3 TetO-ADE2 ho::KanMX::GAL1-TetR*) were used for directed two-hybrid assays and reverse two-hybrid screens essentially as described (22). Strain Y190 was used for quantitative β-galactosidase assays as previously described (25).

Caspase Activation—Jurkat cells were seeded at a density of 1.0×10^6 cells/ml in growth medium + 1 μg/ml cycloheximide. The cells were incubated for 6 h with FasL (2 ng/ml) or TRAIL (50 ng/ml cross-linked with an equal amount of anti-His_6), washed once with phosphate-buffered saline, and then harvested in Triton X-100 lysis buffer then analyzed by immunoblot.

Modeling of FADD Mutations—Free energies were obtained by using the thermodynamic cycle of (wild type protein − mutant protein) − (wild type residues − mutant residues). To determine the free energy for each mutant state and the wild types, the solvated energy was computed from snapshots taken from a 500 ps Molecular Dynamics simulation (one for each mutant/wild type) and averaged over each trajectory. Residue energies were obtained by taking the protein simulations, deleting all of the protein except the mutated residue(s) and computing the solvated energy for each snapshot. The generalized born molecular volume with surface area (26, 27) approach was used to obtain the solvation energy and added to the CHARMM22 (28) force field energy to obtain the solvated energy for each snapshot.

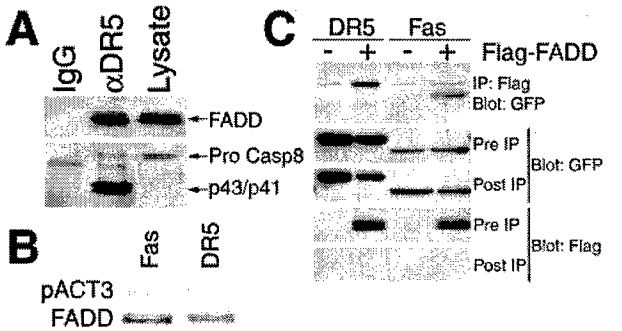

FIG. 1. **FADD binds directly to DR5.** *A*, BJAB cells were stimulated for 30 min with nonspecific IgG or an agonistic DR5 antibody (αDR5), and the endogenous DR5 DISC was precipitated. Both FADD and processed caspase-8 co-precipitate in cells stimulated with αDR5 but not in cells stimulated with IgG. *B*, the cytoplasmic domains of Fas/CD95 or DR5 fused to the Gal4 DNA-binding domain were tested for interaction with FADD in a directed two-hybrid assay. A color change indicates that FADD is able to interact with both Fas/CD95 and DR5 in yeast. *C*, immunoprecipitation (*IP*) experiments were performed to test for interaction between FADD and DR5 or Fas/CD95 *in vivo*. Both GFP-DR5 and GFP-Fas precipitated with FLAG-FADD, indicating a direct interaction. Whole cell lysates were blotted with anti-FLAG and anti-GFP to show equal transfection.

RESULTS

FADD Binds Directly to DR5—We performed DR5 DISC immunoprecipitations to see whether FADD precipitated in the endogenous DR5 DISC. BJAB cells were stimulated for 30 min with either nonspecific IgG or an agonistic monoclonal antibody against DR5 (αDR5), and the DR5 DISC was precipitated. Consistent with previous reports (19, 20), we were able to detect both FADD and caspase-8 in cells stimulated with αDR5 but not in cells stimulated with IgG (Fig. 1*A*).

Although it has been suggested that FADD binds directly to DR5 (21), other reports indicate that an adaptor protein such as TRADD (29) or DAP3 (23) might be involved in recruiting FADD to the DR5 DISC. We therefore used a yeast two-hybrid assay to test for interaction between the cytoplasmic domain of DR5 and full-length FADD. Fas/CD95 was used as a positive control because it is known to interact directly with FADD (30). Both Fas/CD95 and DR5 interacted with FADD in yeast, suggesting that DR5 is recruited directly to the activated TRAIL receptor complex (Fig. 1*B*).

To test whether FADD could bind directly to DR5 in mammalian cells, we performed immunoprecipitation experiments with full-length FADD and the cytoplasmic domains of DR5 and Fas/CD95. Empty FLAG vector or FLAG-tagged FADD was transfected into HeLa cells with GFP-tagged Fas/CD95 or DR5 cytoplasmic domains. FLAG complexes were immunoprecipitated, and interaction was detected by immunoblotting for GFP. As shown in Fig. 1*C*, both Fas/CD95 and DR5 co-precipitated with FLAG-FADD but not with empty vector. We also observed a modest decrease in the amount of GFP-DR5 remaining in the lysate after immunoprecipitation. We conclude that similar to the Fas/CD95 model, FADD binds directly to DR5.

The Death Effector Domain of FADD Modulates Binding to DR5—Using a reverse two-hybrid strategy, we previously identified mutations in the DED of FADD that prevent binding to Fas/CD95 (22). These mutations do not disrupt overall protein

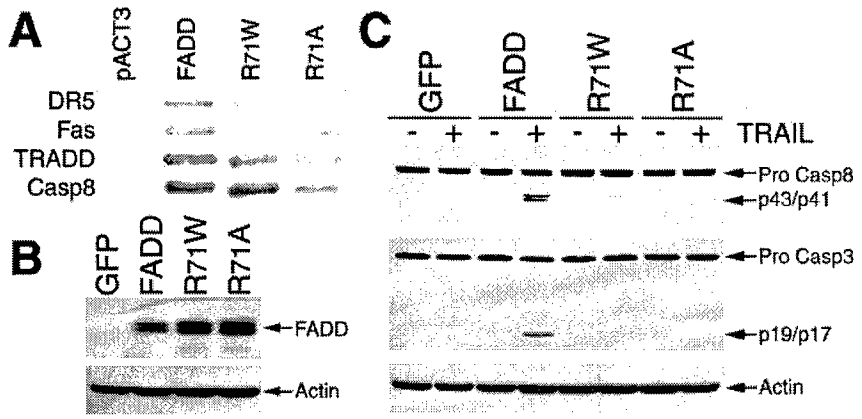

FIG. 2. **The DED of FADD regulates binding to DR5.** *A*, a directed yeast two-hybrid assay was used to test for interaction of DR5, Fas, TRADD, and caspase-8 with empty vector (pACT3), wild type FADD, or FADD DED mutations. Changes in arginine 71 to either alanine or tryptophan prevented interaction with both DR5 and Fas/CD95 while retaining interaction with TRADD and caspase-8. *B*, FADD-deficient Jurkat cells were stably transfected with GFP vector, wild type FADD, or FADD mutants. The level of FADD protein was determined by immunoblot. *C*, stable Jurkat cells were left untreated or stimulated with TRAIL, and caspase-8 and caspase-3 processing was measured by immunoblot. Only cells expressing wild type FADD showed any processing, indicating that arginine 71 is required for FADD binding to DR5.

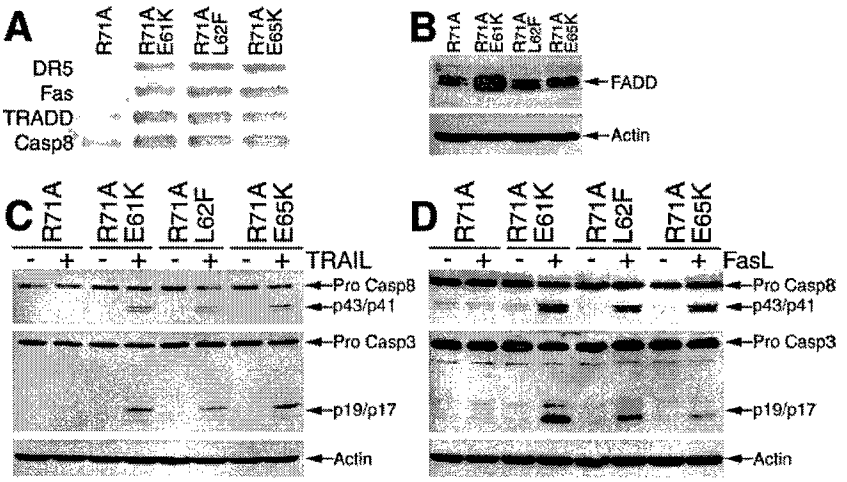

FIG. 3. **Helix 5 of the DED regulates binding of FADD to DR5 and Fas/CD95.** *A*, second site mutations in FADD R71A of glutamates 61 to lysine (R71A E61K), leucine 62 to phenylalanine (R71A L62F), and glutamate 65 to lysine (R71A E65K) restore binding to both DR5 and Fas/CD95 as measured in a directed yeast two-hybrid assay. *B*, FADD expression in cells stably transfected with the second site FADD mutations was determined by immunoblot. Jurkat cells expressing the second site FADD mutations were treated with TRAIL (*C*) or FasL (*D*), and caspase-8 and caspase-3 processing was detected by immunoblot. Each of the second site mutations rescued DR5 and Fas/CD95-induced caspase processing.

structure because the mutated proteins still bind both TRADD and caspase-8 and are able to transduce caspase signaling in response to TNFα in mammalian cells (22). Using a directed yeast two-hybrid assay, we tested these FADD mutations to see whether they affected binding to DR5. A mutation in FADD at arginine 71, which is located in the loop between helices 5 and 6 of the DED (31), to either tryptophan or alanine prevented binding to DR5 (Fig. 2*A*). This suggests that similar to Fas/CD95, the DED of FADD participates in binding to DR5.

To test whether FADD DED mutations could transduce TRAIL signaling, we stably expressed GFP, FADD, FADD (R71W), or FADD (R71A) in FADD-deficient Jurkat cells (24). Jurkat cells express very little DR4 so almost all TRAIL signaling is through DR5 (19). FADD-deficient Jurkat-GFP cells do not express any FADD protein that can be detected by immunoblotting, and the expression level of wild type FADD and the FADD mutations was similar between each cell line (Fig. 2*B*). The earliest signaling event after ligand binding is the recruitment of FADD and processing of caspase-8; FADD mutations that cannot bind to DR5 should therefore be unable to rescue TRAIL-induced caspase-8 processing. These cells were treated with TRAIL, and caspase-8 processing was measured by immunoblot. The cells expressing wild type FADD showed cleavage of caspase-8 in response to TRAIL, whereas cells expressing GFP or the FADD DED mutations did not (Fig. 2*C*). We also measured caspase-3 processing because it is cleaved and activated by caspase-8. Caspase-3 was cleaved only in cells expressing wild type FADD (Fig. 2*C*). Therefore, FADD proteins containing the DED point mutations that prevent binding to DR5 cannot rescue the phenotype associated with FADD deficiency. These data indicate that the binding phenotype observed in yeast correlates with signaling ability in mammalian cells and the DED of FADD modulates binding to DR5.

Mutations That Restore Binding to DR5 Are Located in the DED of FADD—We next sought to identify secondary compensating mutations that would restore binding of FADD DED mutations to DR5. Using a forward two-hybrid approach, we performed a second round of random mutagenesis on FADD (R71A) and screened for second site mutations that restore the binding activity of FADD (R71A) (22). The second site mutations were located in helix 5 of the FADD DED: glutamate 61 to lysine, leucine 62 to phenylalanine, and glutamate 65 to lysine. These mutations restored binding of FADD (R71A) to both DR5 and Fas/CD95 (Fig. 3*A*).

We introduced FADD molecules with these double mutations into FADD-deficient Jurkat cells (Fig. 3*B*) and measured caspase processing after treatment with TRAIL. Cells expressing FADD (R71A) did not show caspase-8 or caspase-3 processing when treated with TRAIL, whereas cells expressing FADD (R71A) along with a second site compensating mutation in the DED showed strong TRAIL-induced caspase cleavage (Fig. 3*C*). Thus, DR5-induced processing of caspase-8 and caspase-3 is prevented by mutations in the DED of FADD and second site mutations that are also in the DED restored processing. Fur-

thermore caspase processing occurs only in response to treatment with the ligand, indicating that it is in response to receptor activation. The same second site mutations also restored Fas/CD95-induced caspase cleavage (Fig. 3*D*). These data suggest that FADD uses the same surface of the DED, specifically helix 5, to bind both DR5 and Fas/CD95.

Full-length FADD Binds Better to DR5 than the Death Domain Alone—Dominant negative FADD, which consists of the DD alone, can inhibit signaling through TRAIL, indicating that the DD is sufficient for binding to DR5 when overexpressed (14). Because the DED is important for the interaction between DR5 and FADD, we reasoned that full-length FADD might bind better than the DD alone. Constructs expressing either the FADD-DD or full-length FADD were tested for interaction with DR5 in yeast. To measure this interaction we used β-galactosidase assays, which allow us to quantitate each interaction. We observed about a 20% increase in binding of DR5 to full-length FADD compared with the DD alone (Fig. 4*A*). We performed immunoprecipitation experiments with FLAG-tagged DR5 and GFP-tagged FADD or FADD-DD to measure this interaction in mammalian cells. Full-length FADD co-precipitated with DR5 to a much greater extent than the death domain alone (Fig. 4*B*). Thus, both the DD and the DED of FADD contribute to the interaction with DR5.

The Death Domain of FADD Can Discriminate between DR5 and Fas/CD95—Because all of our data indicate that binding of FADD to DR5 and Fas/CD95 is very similar, we attempted to identify residues in FADD that are required for binding to one receptor but not the other. We performed a reverse two-hybrid screen to identify mutations in FADD that prevent binding to Fas/CD95 but retain binding to DR5. We screened more than 10 million randomly mutated FADD molecules but were able to identify only a single mutation that discriminated between Fas/CD95 and DR5 binding. A change in valine 108 to glutamate (V108E) in the DD of FADD prevents binding to Fas/CD95 but does not alter binding to DR5, TRADD, or caspase-8 (Fig. 5*A*). We also screened for mutations in FADD that prevent binding to DR5 but retain interaction with Fas/CD95 but were unable to find such a mutation. This implies that other than valine 108, the same residues that are required for FADD binding to DR5 are also required for Fas/CD95 binding.

To determine whether FADD (V108E) rescues signaling in response to activation of DR5 or Fas/CD95 in mammalian cells, we introduced this FADD mutation into FADD-deficient Jurkat cells. The expression level of FADD (V108E) along with cells expressing wild type FADD or GFP is shown in Fig. 5*B*. These cells were treated with TRAIL or FasL, and caspase processing was measured by immunoblot. GFP cells did not show caspase processing when treated with TRAIL or FasL, whereas cells expressing FADD showed both caspase-8 and caspase-3 processing (Fig. 5*C*). Cells expressing FADD (V108E) underwent caspase processing in response to TRAIL but not when treated with FasL (Fig. 5*C*). Thus, FADD (V108E) is able to bind DR5 and transduce TRAIL signaling but is unable to bind Fas/CD95 or transduce signaling through FasL.

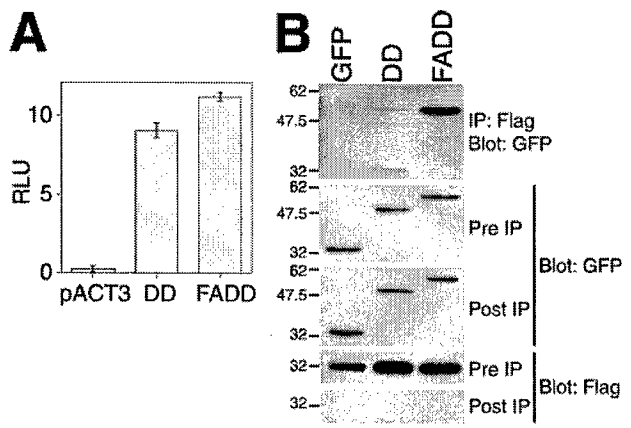

FIG. 4. **DR5 binds to full-length FADD better than the death domain alone.** *A*, the interaction of DR5 with full-length FADD or the DD alone was tested in yeast using quantitative β-galactosidase assays. DR5 showed no interaction with empty vector (pACT3) but was able to interact with both of the FADD constructs. However, the interaction with full-length FADD was about 20% higher than the DD alone. *B*, HeLa cells were transfected with FLAG-DR5 along with GFP, GFP-FADD-DD, or GFP-FADD; FLAG complexes were precipitated, and interaction was measured by immunoblotting for GFP. Full-length FADD interacted with DR5 to a greater extent than the DD alone. *IP*, immunoprecipitation.

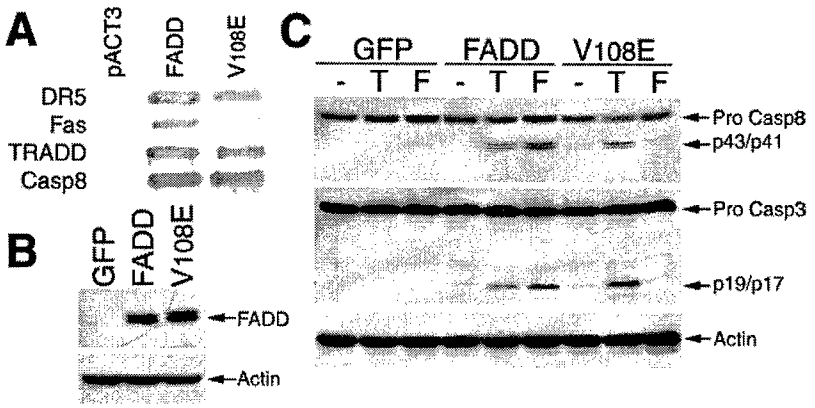

FIG. 5. **Valine 108 is essential for binding of FADD to Fas/CD95.** *A*, a reverse two-hybrid screen identified valine 108 as essential for binding to Fas/CD95. Interaction with other death domain-containing proteins was determined by a directed yeast two-hybrid assay. *B*, the level of exogenous FADD or FADD (V108E) in FADD-deficient Jurkat cells is shown by immunoblot. *C*, Jurkat cells expressing GFP, FADD, or FADD (V108E) were stimulated with TRAIL or FasL, and caspase processing was measured by immunoblot. FADD (V108E) is able to transduce TRAIL signaling but not FasL signaling.

DISCUSSION

We and others have reported that the DED of death domain proteins can regulate binding of the DD (22, 32). Previously we identified mutations in the DED of FADD that prevent binding to Fas/CD95. These FADD mutations did not cause gross conformational changes because binding to TRADD and caspase-8 was still intact. Here we show that mutations in the DED of FADD also prevent binding to DR5. Cells expressing FADD with a mutation in the DED were unable to transduce TRAIL signaling, whereas second site compensating mutations within the DED were able to restore FADD binding to DR5 and rescue TRAIL signaling. Computer modeling indicates that these mutations do not disrupt the overall protein structure because the effect on free energy for most mutations was small (Fig. 6*A*). FADD R71A L62F is the only mutation with a significant change in free energy, but this mutation actually leads to a more stable structure. Interestingly the second site mutations in FADD that rescued binding to DR5 also rescued binding to Fas/CD95, suggesting that FADD uses the same surface of the DED for binding to both receptors.

Both immunoprecipitation and two-hybrid experiments indi-

A

Mutation	ΔG (Kcal/mol)
R71W	2.69 ± 1.24
R71A	2.71 ± 1.30
R71A E61K	2.17 ± 1.27
R71A L62F	-7.59 ± 1.23
R71A E65K	2.21 ± 1.24
V108E	1.84 ± 2.74

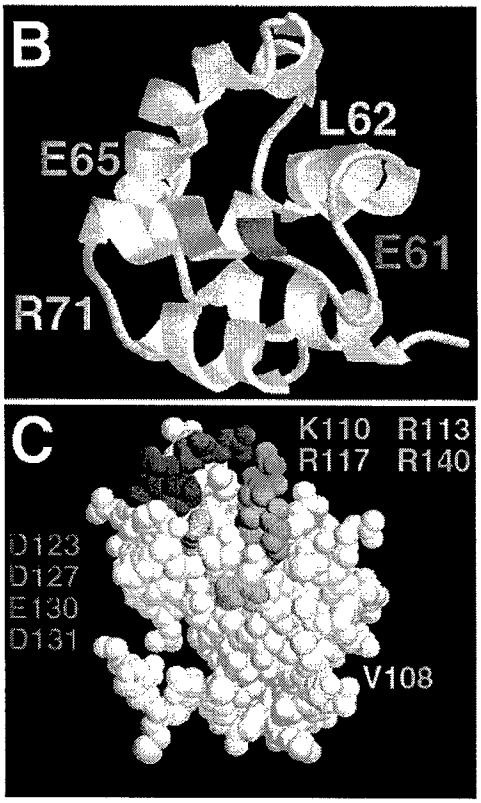

FIG. 6. **The location of FADD mutations.** *A*, the effect of each mutation on the overall structure of FADD was determined by energy minimizations. The change in free energy for each mutation along with the standard error is shown for each mutation. The only mutation to show a large effect was R71A L62F, which led to a more stable structure. *B*, FADD DED mutations were modeled onto the solved structure of the FADD DED (31). Arginine 71, which is required for the FADD-DR5 interaction, flanks helix 5, and the compensating mutations were in helix 5, suggesting a direct role for helix 5 in the FADD-DR5 interaction. *C*, residues previously shown to be important for the FADD-Fas/CD95 interaction (*red* and *blue*) along with valine 108 (*green*) were modeled onto the solved structure of the FADD DD (33).

cate a direct interaction between FADD and DR5. Although it is possible that cellular proteins were mediating this interaction in the immunoprecipitation, our data using FADD DED mutations provide additional evidence for a direct interaction. Mutations of arginine 71 prevented signaling through TRAIL but still allowed for binding to TRADD, suggesting that TRADD is not involved in the FADD-DR5 interaction. In addition, second site mutations that restored interaction of FADD R71A to DR5 in yeast also rescued TRAIL signaling. It would be very unlikely to see the same response in yeast and mammalian cells if there were not a direct interaction between FADD and DR5.

Our data suggest a model in which the DED of FADD comes into direct contact with the receptor. Mutations that disrupt the FADD-receptor interaction were in the loop region-flanking helix 5, and the second site compensating mutations were all in helix 5 of the DED (Fig. 6*B*). Had the effects of the DED on binding to DR5 been allosteric, we would have expected to find compensating mutations in the DD. In addition, we show that DR5 binds more efficiently to full-length FADD than it does to the FADD DD alone. We therefore suggest that in the context of the full-length FADD, helix 5 of the DED comes into direct contact with the receptor.

Although the requirements for FADD binding to DR5 and Fas/CD95 are very similar with regards to the DED, we identified valine 108 in helix 2 of the DD as necessary for binding to Fas/CD95 but dispensable for binding to DR5, TRADD and caspase-8 (Fig. 6*C*). Because we were unable to identify any other mutations in FADD that could discriminate between DR5 and Fas/CD95, we reason that FADD uses the same surface to bind both receptors. Berglund *et al.* (33) identified a patch of charged residues on the surface of FADD that was necessary for binding to Fas/CD95. Valine 108 is near this patch, suggesting that the Fas/CD95-binding surface of FADD is larger than the binding surface for DR5. Taken together, our data indicate that FADD uses the same surface of the DED for binding to DR5 and Fas/CD95, whereas regions within the DD can confer specificity for each receptor.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 50, Issue of December 10, pp. 52479–52486, 2004
Printed in U.S.A.

The C-terminal Tails of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) and Fas Receptors Have Opposing Functions in Fas-associated Death Domain (FADD) Recruitment and Can Regulate Agonist-specific Mechanisms of Receptor Activation*

Received for publication, August 20, 2004, and in revised form, September 24, 2004
Published, JBC Papers in Press, September 27, 2004, DOI 10.1074/jbc.M409578200

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Members of the tumor necrosis factor (TNF) superfamily of receptors such as Fas/CD95 and the TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 induce apoptosis by recruiting adaptor molecules and caspases. The central adaptor molecule for these receptors is a death domain-containing protein, FADD, which binds to the activated receptor via death domain-death domain interactions. Here, we show that in addition to the death domain, the C-terminal tails of DR4 and DR5 positively regulate FADD binding, caspase activation and apoptosis. In contrast, the corresponding region in the Fas receptor has the opposite effect and inhibits binding to the receptor death domain. Replacement of wild-type or mutant DR5 molecules into DR5-deficient BJAB cells indicates that some agonistic antibodies display an absolute requirement for the C-terminal tail for FADD binding and signaling while other antibodies can function in the absence of this mechanism. These data demonstrate that regions outside the death domains of DR4 and DR5 have opposite effects to that of Fas in regulating FADD recruitment and show that different death receptor agonists can use distinct molecular mechanisms to activate signaling from the same receptor.

Members of the tumor necrosis factor (TNF)[1] superfamily of receptors induce a variety of cellular responses including apoptosis, cellular differentiation, and proliferation. A subfamily of these receptors contains a death domain (DD) that is essential for transducing the apoptotic signal. Fas (CD95) is the best characterized member of this family. Binding of Fas Ligand (FasL) to a preformed Fas trimer (1) results in dimerization of two Fas trimers (2) and higher levels of oligomerization (3). These activated receptors signal the apoptotic response by recruiting FADD to the cytoplasmic DD of the receptor to form the death-inducing signaling complex (DISC). NMR studies of the Fas DD indicate that it consists of six anti-parallel α-helices followed by an unstructured tail that negatively regulates Fas activity (4). FADD consists of two distinct domains, a DD, which binds to the DD of Fas, and a death effector domain (DED), which binds to DEDs on caspase-8 and caspase-10 (5) and regulates binding of the DD to the receptor (6). Thus binding of ligand to Fas results in the recruitment of FADD followed by caspases; the induced proximity of two or more initiator caspases results in their dimerization and activation (7, 8). This leads to cleavage to a fully processed, active form of the caspase that can dissociate from the receptor complex. Once activated, these caspases can cleave and activate effector caspases such as caspase-3 to induce the characteristic phenotypes associated with apoptosis.

Signaling by TNF-related apoptosis inducing ligand (TRAIL) is less understood. Interest in TRAIL has arisen because of its reported ability to induce apoptosis in tumor cells without affecting normal cells (9, 10), suggesting that it may be useful for treating cancer. There are two "signaling" receptors, DR4 and DR5, which, similar to Fas, contain DDs followed by a short C-terminal tail. Agonistic antibodies that specifically recognize these receptors provide another way to induce apoptosis (11, 12). In addition, there are two "decoy" receptors DcR1 and DcR2, which have truncated or completely absent death domains (13) and therefore can bind ligand but are unable to transduce the apoptotic signal (14). FADD is recruited to DR4 and DR5 (15, 16) and is required for TRAIL-mediated apoptosis because a dominant-negative form of FADD blocks TRAIL signaling (17) and FADD-deficient Jurkat cells do not undergo TRAIL-induced death (18). Although molecules such as TRADD (19) and DAP3 (20) have been implicated as adaptors in the recruitment of FADD to DR4 and DR5, current models suggest that FADD binds directly to DR4 and DR5 (21) using the same surface of the FADD protein that binds to Fas (22).

Here we show that FADD can bind directly to DR4 and DR5. However, unlike binding of FADD to Fas in which the C-

* This work was supported by grants from the Susan G. Komen Breast Cancer Foundation (to L. R. T.), the United States Army Breast Cancer Research Program (to L. R. T. and A. T.), and National Institutes of Health Grant GM61694 (to J. C. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNF, tumor necrosis factor; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; DD, death domain; DED, death effector domain; DISC, death inducing signaling complex; FADD, Fas-associated death domain; TRADD, TNF receptor-associated death domain; GFP, green fluorescent protein; CHX, cycloheximide; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting.

terminal tail of Fas inhibits FADD recruitment, the C-terminal tails of DR4 and DR5 are required for efficient FADD binding, caspase cleavage and apoptosis. To better understand the importance of the C-terminal tail we made DR5-deficient BJAB cells then introduced wild-type DR5 or a DR5 mutant that lacks the C-terminal tail. While most DR5 stimuli required the C-terminal tail for apoptotic signaling, one agonistic antibody was able to induce apoptosis in the absence of the C-terminal tail, indicating that the process of receptor activation is agonist-dependent. These data suggest a model for TRAIL signaling in which the C-terminal tail that is outside the death domain provides an additional regulatory role.

MATERIALS AND METHODS

Reagents—Antibodies and reagents were purchased from the following sources: caspase-8 and caspase-3 antibodies (Cell Signaling, Beverly, MA), Anti-GFP (Chemicon, Temecula, CA), anti-actin (Sigma), recombinant human TRAIL (R&D Systems, Minneapolis, MN). Agonistic human monoclonal antibodies against DR4 (HGS-ETR1) and DR5 (HGS-ETR2 and R2-A) were provided by Human Genome Sciences (Rockville, MD). mAb631 (agonistic DR5) was from R&D Systems. Human IgG and mouse IgG, as well as anti-mouse and anti-human IgG Fc polyclonal antibodies were from Sigma.

Plasmids—FLAG-tagged versions of DR4 and DR5 in pcDNA3.1 were used for cloning (20). Full-length DR4 and DR5 cDNAs were cloned into the EcoRI and XhoI sites of pcDNA3.1-Puro (+). L334F DR5 was made by site-directed mutagenesis of pcDNA-DR5. Amino acids 1–454 of DR4 and 1–400 of DR5 followed by a TAG stop codon were cloned into the EcoRI and BamHI sites of pcDNA3.1-Puro(−) to generate DR4ΔT (14 amino acids truncation) and DR5ΔT (12 amino acid truncation). pGB14 contains the Gal4 DNA binding domain in a YCp vector. DR4 Cyto (amino acids 272–469), DR4 CytoΔT (amino acids 272–454), DR5 Cyto (amino acids 209–412), DR5 CytoΔT (amino acids 209–400), Fas Cyto (amino acids 177–335) and Fas CytoΔT (amino acids 191–320) were made by PCR from the corresponding cDNAs and cloning the resulting products into pGB14, pBTM-116, pEGFP-C2, and pFLAG-C2. Full-length catalytically inactive caspase-8 was used to generate pGB14-Caspase8. pACT3 plasmids have been described previously (6). Full-length FADD was cloned into the EcoRI and XhoI sites of pFLAG-C2. A more complete description of plasmids, maps, and sequences are available upon request.

TRAIL Receptor Surface Expression—Samples were prepared using the manufacturer's protocol and stained with antibodies against DR5 (R&D systems) or DR4, DcR1, and DcR2 (Alexis, San Diego, CA).

Cell Lines—HeLa cells were maintained in Dulbecco's modified Eagle's medium + 10% fetal bovine serum. BJAB and Jurkat cells were maintained in RPMI 1640 + 10% FBS. BJAB and Jurkat stable cell lines were generated by electroporating pcDNA3.1-Puro constructs and selecting for stable transformants as previously described (6).

DR5-deficient BJAB Cells—DR5-deficient BJAB cells were made by mutagenizing BJAB cells with 2 μg/ml ICR191 (Acros, St. Louis, MO) for 3 h. After a week of recovery, cells were treated with 1 μg/ml CHX and 1 μg/ml cross-linked mAb631 for 3 days. Cells were then washed three times with phosphate-buffered saline and plated in growth media + 1 μg/ml mAb631. After several days, clonal populations were produced by limited dilution. Individual clones were tested for resistance to mAb631 and screened for DR5 surface expression.

Immunoprecipitation—1.5×10^6 HeLa cells were transfected with 2 μg of pEGFP constructs plus 2 μg of FLAG constructs using FuGENE 6 (Roche Applied Science). Cells were lysed in Triton X-100 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1× protease inhibitors), and the soluble fraction was incubated with 30 μl of M2-agarose (Sigma) for 4 h at 4 °C. The beads were washed four times in TBS, and precipitated GFP fusions were detected by immunoblotting.

Two-hybrid Assays—Strains Y190 (Mata his3 ade2 trp1 leu2 gal4 gal80 cyh2, LYS2::Gal1-HIS3 URA3::Gal1-LacZ) and DY6877 (Mata ade2 can1 his3 leu2 lys2 trp1 URA3::8xLexA-LacZ) were used for directed two-hybrid assays.

Cytotoxicity Assays—Jurkat or BJAB cells were seeded in 96-well plates at a density of 1.0×10^6 cells per ml in growth medium + 1.0 μg/ml cycloheximide. Ligands were serially diluted then added to each well. Agonistic DR4 (HGS-ETR1) and agonistic DR5 (mAb631 or HGS-ETR2) were cross-linked with an equal amount of anti-human or anti-mouse Fc before serial dilution. After 22 h, MTS reagent (Promega, Madison, WI) was added to each well and incubated for an additional 2 h. Percent survival was calculated relative to control wells containing no ligand or antibody.

Caspase Activation—Jurkat or BJAB cells were seeded at a density of 1.0×10^6 cells per ml in growth media + 1.0 μg/ml cycloheximide. Agonistic antibodies were cross-linked with an equal amount of anti-mouse or anti-human Fc and unless otherwise stated, used at the following concentrations: mouse or human IgG, mAb631, and HGS-ETR2 at 50 ng/ml; human IgG and HGS-ETR1 (αDR4) at 200 ng/ml; TRAIL was used at 50 ng/ml. Cells were incubated for 6 h, washed once with phosphate-buffered saline, then harvested in Triton X-100 lysis buffer.

DISC Immunoprecipitations—2×10^7 $BJAB^{DR5\ DEF}$ cells expressing the various forms of DR5 were treated with 1.0 μg/ml mAb631 or 0.5 μg/ml ETR2 cross-linked with the appropriate anti-Fc for 30 min. Precipitations were carried out using agarose-protein A/G beads essentially as described (15).

RESULTS

Direct Binding of FADD to DR4 and DR5 Requires the C-terminal Tail of Each Receptor—To test if FADD could bind directly to DR4 and DR5, the cytoplasmic domains of each receptor (Fig. 1A, *DR4 Cyto* and *DR5 Cyto*) were tested for their ability to interact with FADD in a directed two-hybrid assay. As it has been reported that the C-terminal tail of Fas inhibits FADD binding (23), we made constructs that eliminated the corresponding regions of DR4 and DR5 (DR4 CytoΔT and DR5 CytoΔT). As previously reported (24), FADD bound to the cytoplasmic region of Fas only when the C-terminal tail was removed. Surprisingly, the opposite was true for DR4 and DR5; FADD was able to bind only when the C-terminal tails of DR4 and DR5 were intact. This was of interest because removal of the C-terminal tail leaves the six α-helices of the death domains intact, suggesting that interaction of FADD with DR4 and DR5 is regulated by regions outside the receptor death domain.

To test whether the C-terminal tail of DR4 and DR5 is required for interaction with FADD in mammalian cells, we performed co-precipitation assays in HeLa cells using FLAG-tagged FADD and GFP-tagged DR constructs. FLAG complexes were precipitated with an anti-FLAG antibody and interaction of each DR construct was detected by immunoblotting for GFP. Both DR4 Cyto and DR5 Cyto bound to FADD while DR fusions lacking the C-terminal tail did not (Fig. 1C).

If the C-terminal tail of DR4 and DR5 is necessary for FADD binding, then overexpression of the cytoplasmic domains of these receptors should induce apoptosis while DR4 or DR5 lacking the C-terminal tails should not. To test this hypothesis, we transfected GFP-tagged DR5 constructs into HeLa cells. The DR5 cytoplasmic domain (DR5 Cyto) caused cells to round up and die while either GFP alone or the DR5 cytoplasmic domain without the C-terminal tail (DR5 CytoΔT) had no effect (Fig. 1D). Similar results were obtained with GFP-tagged versions of DR4 (data not shown). These data suggest that FADD binding and apoptosis through DR4 and DR5 require the C-terminal tail of the receptor. Surprisingly, this is the opposite of the Fas receptor where the C-terminal tail is inhibitory to FADD binding and receptor function (23).

The C-terminal 14 Amino Acids of DR4 Are Required for Apoptosis and Caspase Activation—To test whether the 14 C-terminal amino acids of DR4 are necessary for transducing the apoptotic signal by receptor activation, we made BJAB cells that stably express GFP and DR4ΔT (removal of amino acids 455–469 from full-length DR4 constituting the C-terminal tail). We hypothesized that DR4ΔT should act as a dominant-negative and inhibit DR4 induced apoptosis because it can interact with endogenous receptors and bind ligand but should inhibit the apoptotic signal as it is unable to bind FADD. We obtained several DR4ΔT clones and the expression level of DR4 for two representative clones is shown in Fig. 2A. Because

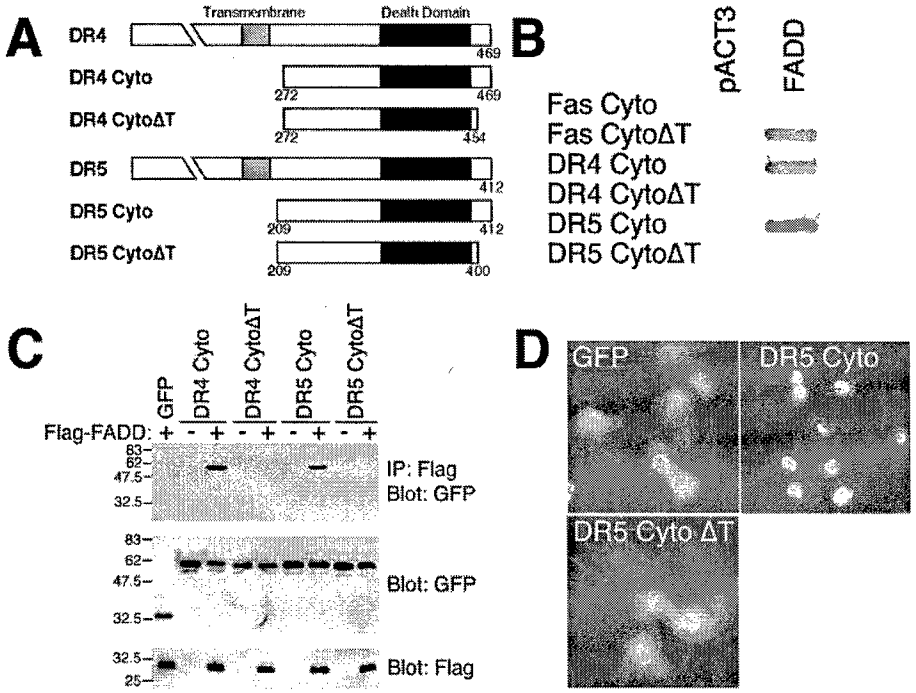

FIG. 1. **The C terminus of DR4 and DR5 binds directly to FADD and is required for induction of apoptosis.** *A*, graphical representation of DR4 and DR5 constructs. The indicated fragments were cloned into GFP or two-hybrid vectors. *B*, binding of Fas, DR4, and DR5 constructs to empty vector (pACT3) or FADD was measured by directed two-hybrid. FADD binds only to Fas when the 15 C-terminal amino acids are removed, but binds to DR4 and DR5 only when the C-terminal tail is present. *C*, HeLa cells were transfected with GFP or GFP-DR5 constructs. After 14 h, green cells expressing DR5 Cyto (*green*) show typical signs of apoptosis while cells expressing GFP alone or DR5 CytoΔT do not, indicating that the C-terminal tail of DR5 is required for apoptosis. *D*, HeLa cells were transfected with GFP-DR constructs along with empty FLAG vector (FLAG) or FLAG-FADD. FLAG complexes were immunoprecipitated and blotted with a GFP-specific antibody. Consistent with the results in two-hybrid assays, only DR4 Cyto and DR5 Cyto but not DR5 CytoΔT or DR5 CytoΔT bind FADD.

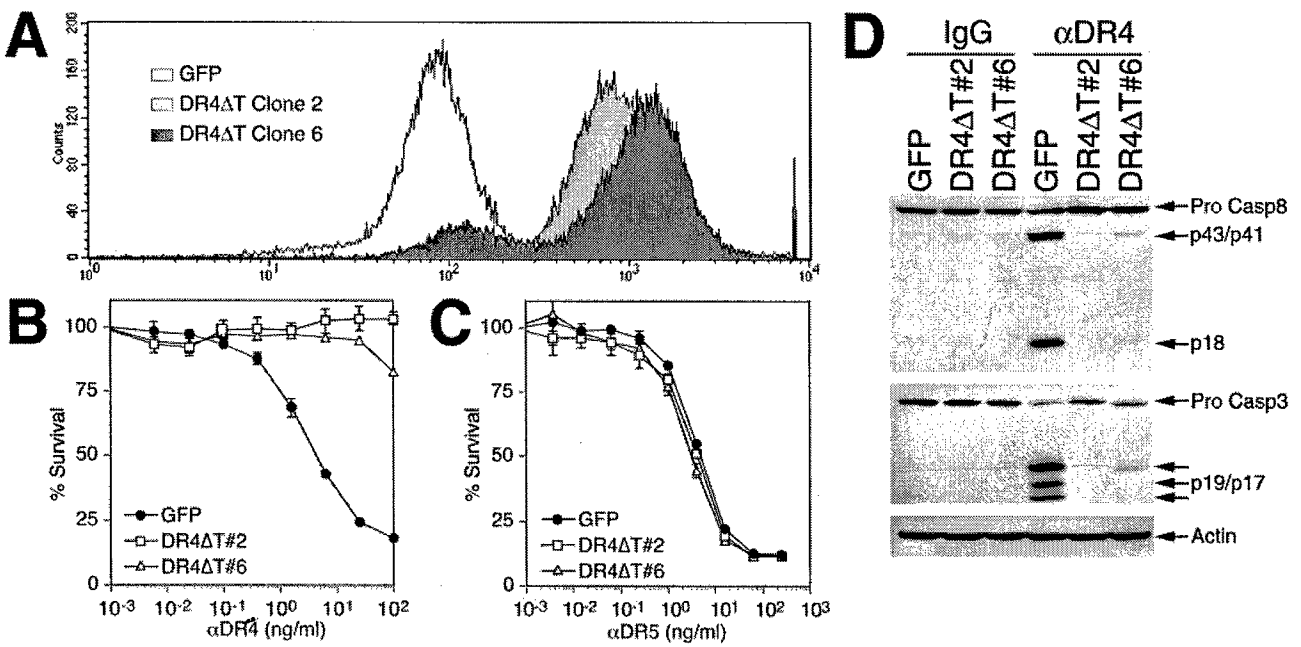

FIG. 2. **Signaling through DR4 requires the 12 C-terminal amino acids in BJAB cells.** *A*, expression level of DR4ΔT in stable transfectants is shown. The expression of DR4ΔT in the two clones is about 10-fold higher than the expression of endogenous DR4. *B*, cells were treated with the indicated amount of αDR4 (HGS-ETR1) and cytotoxicity was measured by MTS. Cells expressing DR4ΔT were completely resistant to αDR4 compared with GFP control cells. *C*, dose responses using an agonistic DR5 antibody, mAb631, show that cytotoxicity was nearly identical between the three cell lines indicating that there were no other defects in apoptotic signaling. *D*, BJAB cells were treated with nonspecific IgG or αDR4 (HGS-ETR1), and caspase processing was measured by immunoblot. Control GFP cells showed caspase-8 and caspase-3 processing in response to αDR4 while DR4ΔT clones did not.

BJAB cells express both DR4 and DR5, we selectively induced TRAIL receptor-mediated apoptosis using agonistic monoclonal antibodies that are specific for each receptor. All agonistic antibodies are IgG1 subtypes and were cross-linked with anti-IgG antibody before treatment. BJAB-GFP and BJAB-DR4ΔT cells were treated with increasing amounts of a DR4

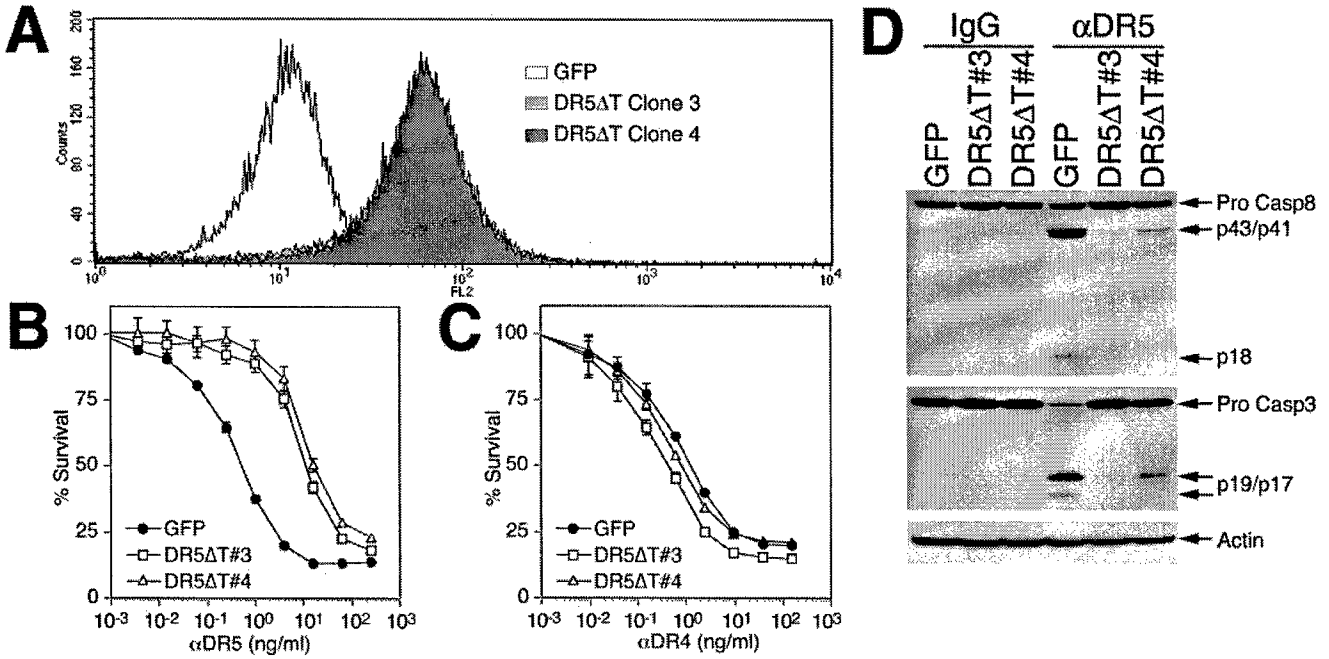

FIG. 3. **Signaling through DR5 requires the 12 C-terminal amino acids in BJAB cells.** *A*, FACS analysis was used to measure the expression level of DR5 and DR5ΔT in stable transfectants. DR5ΔT is expressed at about five times the level of endogenous DR5 compared with GFP control cells. Note that the expression level of DR5ΔT in the two clones is nearly identical. *B*, BJAB cells were treated with the indicated amount of the αDR5 (mAb631) and cytotoxicity was measured by MTS. An approximate 50-fold shift in the dose response indicated that DR5ΔT protects cells against cytotoxicity through DR5. *C*, signaling downstream of the DR5 receptor is still intact because dose responses using αDR4 (HGS-ETR1) show that cytotoxicity was nearly identical between the three cell lines. *D*, BJAB cells were treated with nonspecific IgG or αDR5 and caspase processing was measured by immunoblot. αDR5-stimulated cleavage of caspase-8 and caspase-3 in BJAB-GFP cells, but this activation was inhibited by the expression of DR5ΔT.

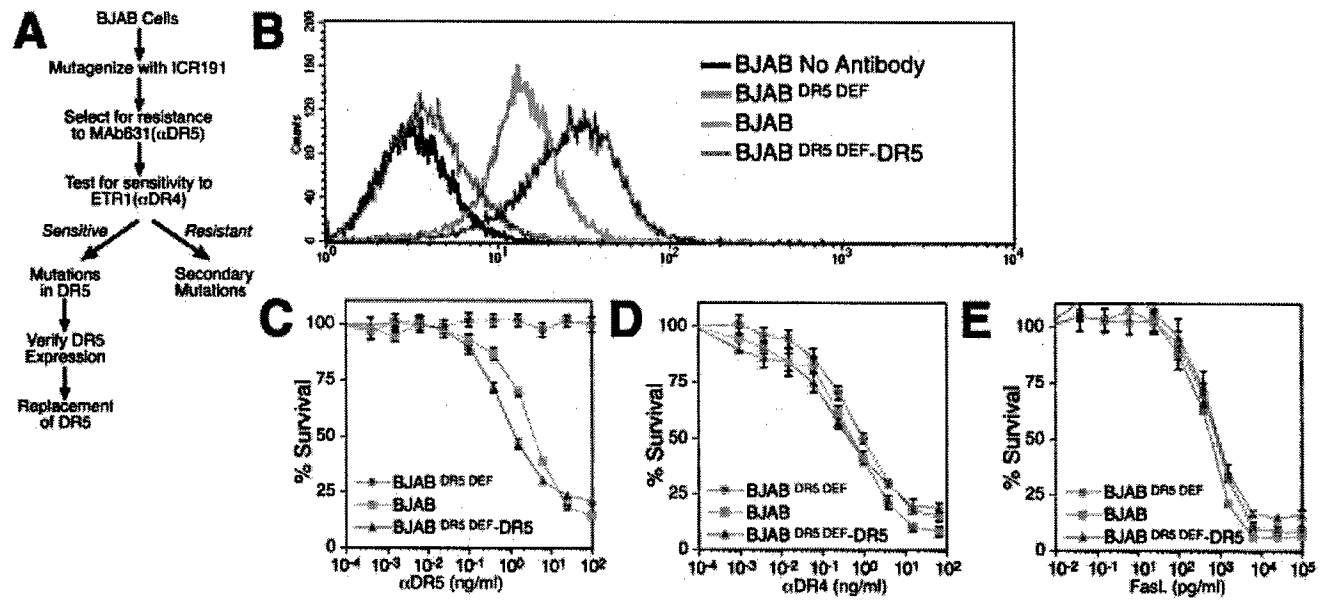

FIG. 4. **Construction of DR5-deficient BJAB cells.** *A*, scheme used to identify DR5-deficient BJAB cells by testing sensitivity to various agonists. *B*, surface DR5 expression in wild-type BJAB cells, DR5-deficient BJAB cells ($BJAB^{DR5\ DEF}$) or DR5-deficient BJAB cells stably expressing DR5 ($BJAB^{DR5\ DEF}$-DR5) was determined by FACS. *C*, each cell line was treated with αDR5 (mAb631), and cytotoxicity was measured. $BJAB^{DR5\ DEF}$ cells are resistant to αDR5 and replacement of DR5 into these cells renders them sensitive. All cell lines are equally sensitive to αDR4 (*D*) and FasL (*E*) indicating that the only defect in DR5 induced apoptosis in $BJAB^{DR5\ DEF}$ cells is loss of DR5 expression.

agonistic antibody (αDR4) and cytotoxicity was measured. Clones expressing DR4ΔT, which has an intact DD but no C-terminal tail were completely resistant to αDR4 while GFP-expressing cells were sensitive (Fig. 2*B*). To test if this was caused by the stable expression of DR4ΔT and not some other defect in apoptosis, the same cells were treated with an agonistic antibody against DR5 (αDR5). As shown in Fig. 2*C*, the dose responses for αDR5-induced cytotoxicity in cells expressing GFP and DR4ΔT are nearly identical indicating apoptotic signaling downstream of the DR4 receptor is intact.

The immediate effect of ligand binding to a death receptor is the recruitment of FADD and procaspase-8. This leads to caspase-8 activation and proteolytic cleavage (8). If FADD is unable to bind to DR4ΔT, then cells expressing this construct

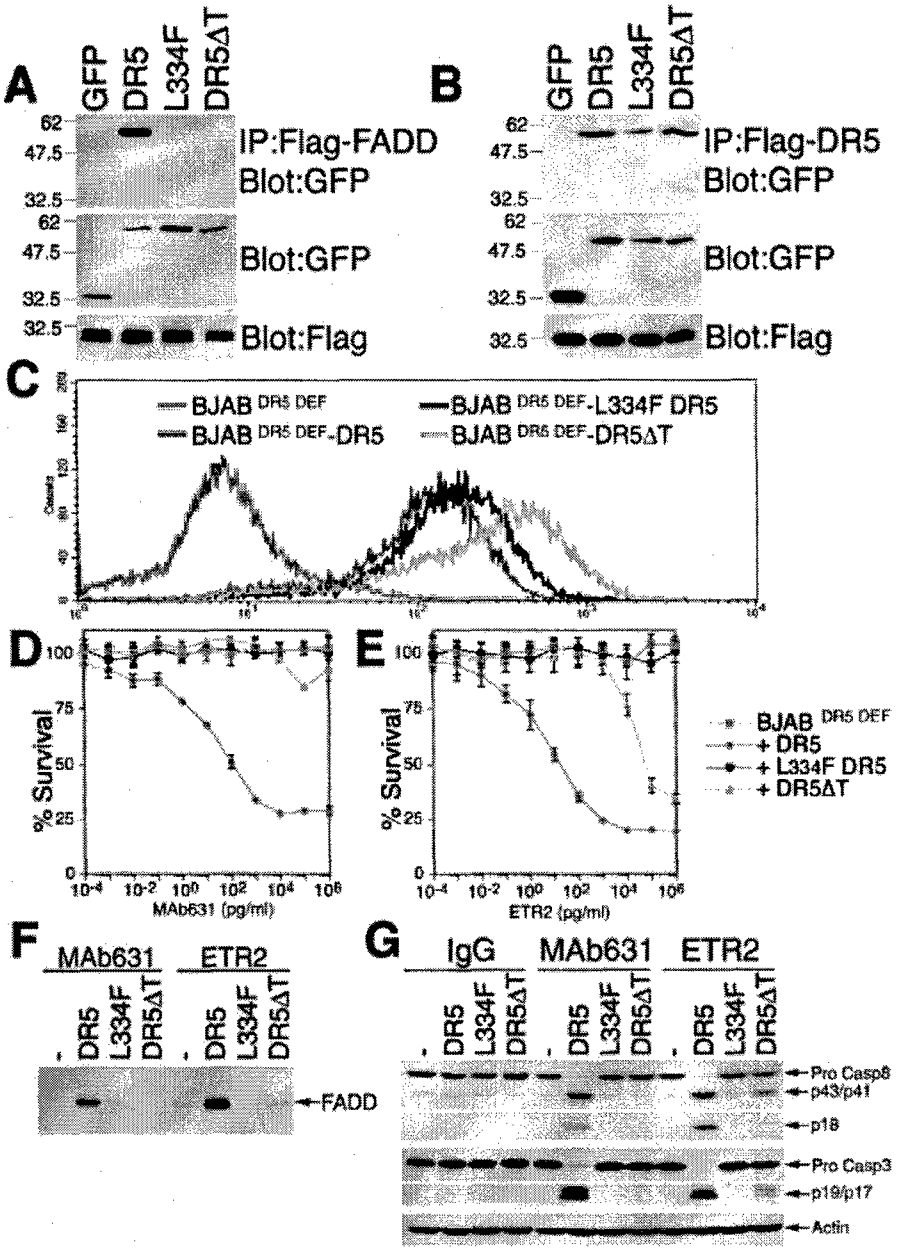

FIG. 5. **The C-terminal tail of DR5 differentially regulates apoptosis in response to various agonistic antibodies.** *A*, HeLa cells were transfected with FLAG-FADD and GFP-tagged DR5 cytoplasmic domain. FLAG complexes were precipitated and immunoblotted for GFP to detect interaction of each DR5 construct with FADD. Wild-type DR5 is able to interact with FADD whereas DR5 (L334F) and DR5ΔT cannot. Whole cell lysates were immunoblotted for GFP and FLAG expression to show equal transfection. *B*, cytoplasmic domain of DR5 fused to FLAG was transfected into HeLa cells with GFP-tagged DR5 or DR5 mutants. FLAG complexes were precipitated, and interaction with DR5 was detected by immunoblotting for GFP. DR5, DR5 (L334F), and DR5ΔT co-precipitate with DR5 indicating that these mutations do not destroy overall protein structure. Whole cell lysates were immunoblotted for GFP and FLAG expression to show equal transfection. *C*, level of surface DR5 expression in BJAB$^{DR5\ DEF}$ cells stably expressing DR5, DR5 (L334F), or DR5ΔT was determined by FACS. *D*, BJAB$^{DR5\ DEF}$ cells expressing the various DR5 constructs were treated with mAb631 and cytotoxicity was measured. Only cells expressing DR5 are sensitive to mAb631. *E*, both DR5- and DR5ΔT-expressing cells are sensitive to HGS-ETR2, but cells expressing DR5 (L334F) are still resistant indicating that the differential sensitivity to various DR5 agonists is caused by the tail and not simply the inability to bind FADD. *F*, BJAB$^{DR5\ DEF}$ cells or cells expressing DR5, DR5 (L334F), or DR5ΔT were stimulated for 30 min with mAb631 (1.0 μg/ml) or HGS-ETR2 (0.5 μg/ml). FADD was detected in the endogenous DISC only in DR5-expressing cells when treated with mAb631 but was in both the DR5 and the DR5ΔT DISC when cells were stimulated with ETR2. *G*, same cells were treated with 1 μg/ml of IgG, mAb631, or 500 ng/ml HGS-ETR2 and caspase processing was determined by immunoblot. Caspase 8 was processed into its p43/p41 form and the p18 active subunit and caspase-3 was processed into its p19/p17 active form in BJAB$^{DR5\ DEF}$ cells expressing DR5 when treated with mAb631 or HGS-ETR2. BJAB$^{DR5\ DEF}$ cells expressing DR5Δ3 showed caspase processing only in response to HGS-ETR2.

should not process caspase-8 upon DR4 receptor activation. BJAB cells were treated with either nonspecific IgG or αDR4, and caspase-8 processing was examined by immunoblotting. Caspase-8 was processed from its proform into its p43 and p41 cleavage products as well as the p18 active subunit in BJAB cells expressing GFP whereas clones expressing DR4ΔT showed only very small amounts of the p43/p41 subunit (Fig. 2*D*). Caspase-3 processing was also examined as it is cleaved and activated by caspase-8. GFP-expressing cells showed high amounts of caspase-3 processing in response to αDR4 while DR4ΔT expressing clones showed very little. We conclude that the 14 C-terminal amino acids of DR4 that are outside the death domain are required for efficient FADD binding and activation of the apoptotic cascade.

The C-terminal 12 Amino Acids of DR5 Are Required for Apoptosis and Caspase Activation—To test whether the C-terminal tail is required for DR5 function, we made BJAB cells that stably express DR5ΔT (full-length receptor lacking amino acids 401–412 constituting the C-terminal tail). The expression level of DR5 for two representative clones as well as GFP control cells is shown in Fig. 3*A*. Clones expressing DR5ΔT showed increased resistance to αDR5 compared with GFP cells (Fig. 3*B*) as indicated by an approximate 50-fold shift in the dose response. Apoptotic signaling was otherwise intact because these cells were still sensitive to αDR4 (Fig. 3*C*). The same cells were treated with nonspecific IgG or αDR5 and caspase processing was examined by immunoblotting. As shown in Fig. 3*D*, both caspase-8 and caspase-3 were processed into their active subunits in BJAB-GFP cells but not in the clones expressing DR5ΔT when stimulated with αDR5. We therefore conclude that similar to DR4, the C-terminal tail of DR5 is required for cytotoxicity and caspase processing.

Identification of DR5-deficient BJAB Cells and Complementation with DR5 Transgene—Because experiments using dominant negative proteins rely on overexpression, we examined the effects of DR5ΔT in a DR5-deficient background. Because no DR5-deficient cell lines have been identified (13), we made DR5-deficient BJAB cells. BJAB cells were randomly mu-

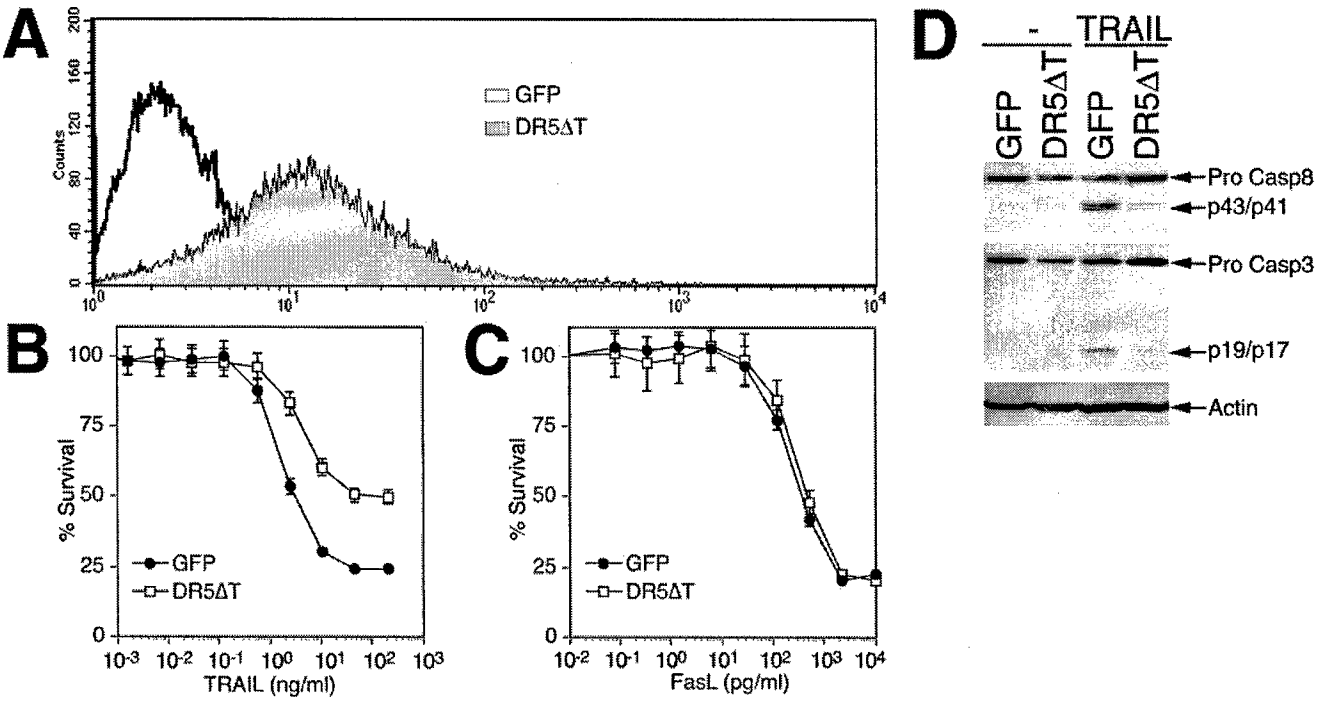

FIG. 6. **TRAIL signaling requires the C-terminal tail of DR5 in Jurkat cells.** *A*, expression level of DR5 in Jurkat GFP cells or a clone stably expressing DR5ΔT was determined by FACS analysis. *B*, GFP or DR5ΔT cells were treated with the indicated amounts of TRAIL. DR5ΔT protects cells from cytotoxicity induced by TRAIL as indicated by a shift in the dose curve. *C*, when treated with FasL, there is no difference in the dose response of the various Jurkat cells indicating that downstream apoptotic pathways are still intact. *D*, Jurkat cells were treated with TRAIL and caspase processing was measured by immunoblot. Both caspase-8 and caspase-3 are activated in response to TRAIL in Jurkat cells expressing GFP, but not in the clone expressing DR5ΔT.

tagenized with ICR191 followed by selection for clones resistant to αDR5-induced death (Fig. 4*A*). Because resistance could be caused by loss of DR5 or some other component of the apoptotic pathway such as FADD or caspase-8, each clone was also tested for sensitivity to αDR4 and FasL. We reasoned that clones with no functional DR5 receptor should be sensitive to these two stimuli while those with defects in downstream signaling would be resistant. The subset of clones sensitive to DR4 and FasL were screened for loss of DR5 surface expression by FACS. A clone we designated BJAB$^{DR5\ DEF}$ did not express any detectable DR5, was resistant to mAb631 and sensitive to both αDR4 and FasL (Fig. 4, *B–E*).

If the defect in BJAB$^{DR5\ DEF}$ cells is loss of DR5 expression rather than loss of receptor transport as has been recently observed (25) or unrelated anti-apoptotic defects, then replacement of DR5 into these cells should restore DR5 signaling. We introduced DR5 into BJAB$^{DR5\ DEF}$ cells and identified a clone that stably expressed surface DR5 at levels comparable to wild-type BJAB cells as determined by FACS analysis (Fig. 4*B*). These cells showed similar cytotoxicity compared with wild-type BJAB cells when treated with αDR5, αDR4, and FasL (Fig. 4, *C–E*). Because replacement of DR5 into BJAB$^{DR5\ DEF}$ cells completely restores DR5 induced signaling, we conclude that the only DR5 signaling defect in BJAB$^{DR5\ DEF}$ cells is lack of DR5 expression.

The C-terminal Tail of DR5 Is Required for Signaling in Response to mAb631 but Not HGS-ETR2—To test whether DR5ΔT had activity in the absence of endogenous DR5 receptor, DR5ΔT was stably expressed in BJAB$^{DR5\ DEF}$ cells. As a control for a DR5 receptor that cannot bind FADD because of a death domain defect, DR5 (L334F) was also expressed in the DR5-deficient background. A mutation of leucine 334 to phenylalanine in the death domain of DR5 is analogous to the *lpr* mutation in Fas, which abolishes receptor function by preventing FADD binding (19). Neither DR5 (L334F) nor DR5ΔT are able to bind FADD as determined by co-immunoprecipitation experiments in mammalian cells (Fig. 5*A*). To test whether these mutations completely destroyed protein function, we tested each DR5 construct for interaction with the cytoplasmic domain of wild-type DR5. DR5 forms homomeric complexes and this association occurs through interaction between the DR5 cytoplasmic domain (26). Each DR5 construct co-precipitated with FLAG-DR5 indicating that the cytoplasmic domains of DR5 (L334F) and DR5ΔT are functionally intact (Fig. 5*B*).

We next made cell lines stably expressing DR5 (L334F) or DR5ΔT. FACS analysis was used to identify clones expressing surface levels of DR5 (L334F) or DR5ΔT similar to BJAB$^{DR5\ DEF}$-DR5 (Fig. 5*C*). Cytotoxicity in BJAB$^{DR5\ DEF}$ cells or clones expressing exogenous DR5, DR5 (L334F) and DR5ΔT was measured after treatment with several DR5 agonistic antibodies. When treated with mAb631, the agonistic DR5 antibody used in previous experiments, BJAB$^{DR5\ DEF}$ cells expressing wild-type DR5 were sensitive whereas cells expressing DR5ΔT or DR5 (L334F) were resistant (Fig. 5*D*). A similar pattern of sensitivity was observed when the same cells were treated with a second DR5 agonistic antibody, R2-A (data not shown). These observations were consistent with our previous data in which removal of the C-terminal tail from DR5 prevented signaling through the receptor. A different result was obtained when we treated cells with a third DR5 agonistic antibody, HGS-ETR2. BJAB$^{DR5\ DEF}$ cells expressing exogenous DR5 or DR5ΔT were sensitive (Fig. 5*E*) although DR5ΔT cells required higher doses of HGS-ETR2. In contrast, cells expressing DR5 (L334F) were completely resistant to HGS-ETR2. These data suggest that the tail of DR5 is not absolutely required for signaling induced by HGS-ETR2 as it is with other DR5 agonistic antibodies. Since DR5 (L334F) cells are resistant to every DR5 agonist but DR5ΔT cells are differentially sensitive we conclude that the death domain is essential while the tail of DR5 mediates sensitivity of the receptor to different agonists.

Because HGS-ETR2 was able to induce apoptosis in BJAB$^{DR5\ DEF}$ cells expressing both DR5 and DR5ΔT, but mAb631 was able to induce apoptosis only in cells expressing DR5, we reasoned that these two antibodies might differentially recruit FADD to the truncated DR5 receptor. To test this hypothesis we performed DISC immunoprecipitation experiments with BJAB $^{DR5\ DEF}$ cells or cells expressing DR5, DR5 (L334F), or DR5ΔT and immunoblotted for FADD. mAb631 recruited FADD to the DR5 receptor only in BJAB$^{DR5\ DEF}$ cells expressing wild-type DR5 (Fig. 5*F*). However, ETR2 was able to recruit FADD in cells expressing DR5 and cells expressing DR5ΔT. FADD was recruited to a lesser extent in cells expressing DR5ΔT compared with DR5 consistent with the dose response data in which DR5ΔT-expressing cells were sensitive to ETR2, but to a lesser extent than DR5-expressing cells.

Next, we assessed caspase processing in response to saturating doses of mAb631 (1 μg/ml) and HGS-ETR2 (500 ng/ml). mAb631 was able to induce caspase-8 and caspase-3 processing only in BJAB$^{DR5\ DEF}$ cells expressing DR5 (Fig. 5*G*). Consistent with the dose response data, treatment with HGS-ETR2 resulted in the processing of caspase-8 and caspase-3 in BJAB$^{DR5\ DEF}$ cells expressing DR5, and cells expressing DR5ΔT. These data suggest that the C-terminal tail of DR5 is necessary for FADD recruitment through most stimuli. Because other stimuli such as the HGS-ETR2 antibody can overcome this defect and recruit FADD to induce caspase activation, diverse agonists to the DR5 receptor might activate the receptor through different mechanisms.

TRAIL Signaling through DR5 Is Prevented by Removal of the C-terminal 12 Amino Acids—We next wished to assess whether TRAIL activation of DR5 requires the C-terminal tail similar to mAb631 or whether TRAIL can activate the receptor independent of the C-terminal tail like HGS-ETR2. To test any inhibitory effect associated with removal of the C-terminal tail in response TRAIL, we made Jurkat cells that stably express GFP or DR5ΔT. Jurkat cells express very low levels of DR4 (12) so almost all TRAIL signaling is through DR5. We identified several clones that expressed high levels of exogenous DR5ΔT all with similar responses to TRAIL; the expression level of DR5ΔT in a representative clone is shown in Fig. 6*A*. Jurkat cells expressing DR5ΔT showed decreased cytotoxicity compared with cells expressing GFP when treated with TRAIL (Fig. 6*B*). Apoptosis signaling was still intact in DR5ΔT Jurkat cells because all cell types were equally sensitive to death from FasL (Fig. 6*C*). When treated with TRAIL, Jurkat-GFP cells showed high levels of caspase-8 and caspase-3 processing, whereas cells expressing DR5ΔT showed very little (Fig. 6*D*). These data indicate that TRAIL signaling through DR5 requires the C-terminal tail similar to signaling with mAb631.

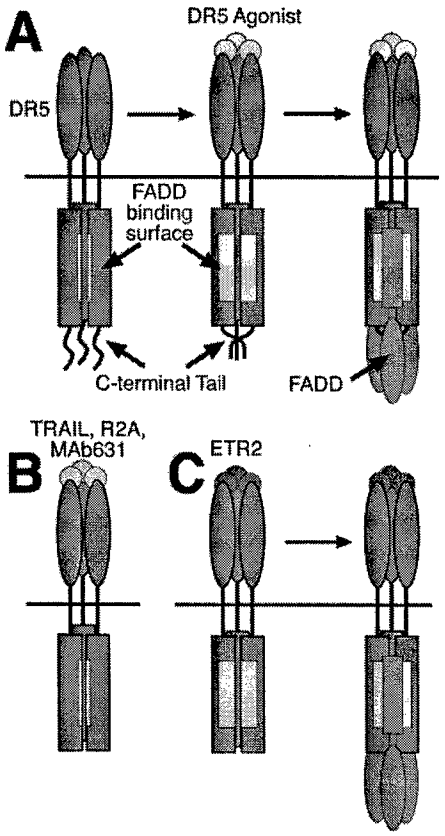

FIG. 7. **The C-terminal tail of DR5 regulates the recruitment of FADD.** *A*, when ligand is not bound, DR5 is held in a trimeric complex through interactions between the cytoplasmic domain of each receptor. In this state, the FADD binding surface on DR5 is not accessible to FADD. Binding of ligand to a DR5 trimer results in a conformational change in the trimer exposing the FADD binding surface. The change is facilitated by the C-terminal tail and allows FADD to bind. *B*, when the tail is absent, binding of agonists such as TRAIL or mAb631 cannot cause the conformational change required to expose the FADD binding surface. *C*, binding of some agonists such as HGS-ETR2 can cause the conformational change that leads to FADD recruitment even when the tail is not present.

DISCUSSION

Although there is much interest in TRAIL signaling because of its reported ability to kill tumor cells through FADD-dependent apoptosis while not affecting normal cells, the mechanism by which FADD is recruited to DR4 and DR5 is unclear. While some reports implicate TRADD (19) or DAP3 (20) as an adaptor molecule to link FADD to DR4 and DR5, our data presented here and in a recent publication (22) suggests a model in which FADD can bind directly to the receptor.

Because the C-terminal tail of Fas (amino acids 321–335) inhibits FADD binding to Fas, and FADD uses a similar surface for binding to both DR5 and Fas (22), we thought that removal of the corresponding regions of DR4 and DR5 would enhance interaction of FADD with these receptors. Interestingly, the opposite result was obtained and removal of the C-terminal tails (14 amino acids from DR4 and 12 amino acids from DR5) inhibits FADD binding. Although Fas and TRAIL receptors are similar in that FADD is recruited directly, this suggests that the requirements for FADD binding are different. Removal of the C-terminal tails from DR4 and DR5 leaves the DD structurally intact because DR4 and DR5 mutants that lack the C-terminal tail are still able to bind their full-length counterpart. Thus, unlike the situation with Fas, the DDs of TRAIL receptors are not sufficient for FADD binding. Fas is the only mammalian death receptor with a solved structure for the DD (4), and it has therefore served as a model for all other death receptors. Although a part of the C-terminal tail of Fas was removed in the solved structure, Huang *et al.* (4) observed that the region encompassing the death domain was well defined while the carboxyl terminal amino acids were disordered. Structural studies of the DR4 and DR5 intracellular domain will be important and may demonstrate that the corresponding C-terminal region of TRAIL receptors is more structured.

DR4 and DR5 can form homomeric or heteromeric complexes (15), and this association occurs through interaction between the cytoplasmic domains of each receptor (26). Thus, one would expect that the tail-less DR4 receptor might partially block signaling through DR5 because this heteromeric complex should not bind FADD through the DR4ΔT cytoplasmic domain. However, we observed that signaling through DR5 that was activated by the DR5-specific antibody was unaltered in

cells expressing DR4ΔT. The same was true for cells expressing DR5ΔT; responses to αDR4 were similar to those of GFP-expressing cells. This suggests that heteromeric complexes do not play an important role in apoptotic signaling through DR4 and DR5. In agreement with this view, Kischkel *et al.* (15) observed that there were fewer heteromeric complexes than homomeric complexes when the endogenous TRAIL DISCs were examined by immunoprecipitation.

Quantitative differences between agonistic antibodies and FasL have been observed for the Fas receptor (27). In this case the anti-apoptotic protein Bcl-xL could block death induced by Fas agonistic antibodies but not FasL suggesting that different agonists can signal through distinct mechanisms. Our data uncover a potential mechanism for such differential sensitivity in TRAIL signaling because different agonistic antibodies can display different requirements for regions in the intracellular domain of DR4 or DR5. Thus, binding of different agonists to the extracellular domain causes subtly different conformational changes in the intracellular domain that define the FADD binding surface of the receptor.

We propose a model for TRAIL signaling in which the C-terminal tail plays a regulatory role in the recruitment of FADD to DR5 (and DR4). In this model, DR5 exists as a preformed trimer held together by interactions between the cytoplasmic domains of each receptor. In the absence of bound ligand the FADD binding surface of the trimers is not accessible to FADD (Fig. 7*A*). Binding of TRAIL or an agonistic antibody to the receptor trimer causes a conformational change that is normally facilitated by the C-terminal tail. This alteration exposes the FADD binding surface leading to the recruitment of FADD and initiation of the apoptotic cascade. In the absence of the C-terminal tail, binding of some ligands such as TRAIL or mAb631 is not sufficient to trigger this conformational change (Fig. 7*B*), FADD cannot be recruited and there is no apoptotic signal. However, some agonists such as HGS-ETR2 are able to overcome this layer of regulation and recruit FADD even in the absence of the C-terminal tail (Fig. 7*C*). These data indicate that activation of TRAIL receptors has mechanistic differences compared with activation of the Fas receptor and raise the possibility that it will be feasible to activate subtly different signaling mechanisms from the same receptor using different agonists.

Acknowledgments—We thank Dr. Marcus Peter for providing BJAB cells and Minna Poukkula for help with DR4 and DR5 receptor staining.

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Journal of Biological Sciences 5 (1): 33-37, 2005
ISSN 1727-3048

Death Domain Interactions in Death Receptor Signaling

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Apoptosis is a form of programmed cell death that eliminates damaged, infected, or extraneous cells by the activation of a cell suicide program[1]. Induction of apoptosis is tightly regulated and disassembles the cell from within to avoid eliciting an inflammatory response. Apoptosis plays a critical role in the development and maintenance of multicellular organisms. In addition defective apoptosis is thought to be essential in the development of cancer[1] and is also involved in the genesis and treatment of other diseases[2]. For example, it is thought that cancer originates from cells receiving inappropriate growth signals as well as failing to receive or properly respond to appropriate signals to die while many cancer treatments work by inducing apoptosis. Therefore, there has been significant interest in the characterization of apoptotic pathways over the past 10-20 years.

There are two well-characterized pathways (and possibly several others) that regulate the induction of apoptosis[3]. Both of these pathways are depend upon a family of cysteine proteases termed caspases, which function to systematically dismantle the cell through their progressive proteolytic activity. Caspases fall into two main classes, initiator caspases and effector caspases. Initiator caspases can autoactivate and then subsequently cleave downstream substrates including effector caspases to activate the caspase cascade and cause the demise of the cell. The intrinsic apoptotic pathway is activated in response to cellular stress and relies upon disruption of the mitochondrion and release of cytochrome c. Upon release, cytochrome c promotes caspase 9 activation by forming a complex with APAF-1, procaspase 9 and dATP termed the apoptosome. In contrast, the extrinsic apoptotic pathway relies upon the engagement of a death receptor. There are currently 6 known death receptors including CD95 (FAS), TNF receptor 1 (TNFR-1), DR3/APO-3/TRAMP, TRAIL receptor 1/DR4, TRAIL-R2/DR5 and DR6. Death receptors are transmembrane receptors that activate apoptosis in addition to other signaling pathways in response to ligand binding. The activated receptor, which is a trimer[4], undergoes a conformational change facilitating the recruitment of proteins to the intracellular portion of the receptor, which is a death domain[5], a conserved approximately 80 amino acid domain that is characteristic of all members of the superfamily. The receptor death domain can bind to other death domain containing proteins to facilitate downstream signaling. While the initiator caspase for the intrinsic apoptotic pathway is thought to be caspase 9, the initiator caspase for the extrinsic apoptotic pathway is caspase 8 or sometimes a very similar caspase called caspase 10. While caspases 8 and 10 serve as the initiator caspases in response to death receptor activation, there is an amplification loop that is required for efficient death receptor induced death in certain cell types. This loop relies on cleavage of Bid[6], which translocates to the mitochondria to activate the intrinsic apoptotic pathway and caspase 9. Therefore although the two caspase activation pathways are separate, they can be linked in at least some cells via Bid. In both cases however the key regulatory event is the activation of caspase 8, which arises because of interactions between death domain-containing proteins. In this review, we have discussed what is known about how these interactions are regulated.

The death domain superfamily: The death domain superfamily is composed of a group of proteins possessing a Death Domain (DD) and two related domains called a Death Effector Domain (DED), or a Caspase Activation Recruitment Domain (CARD)[5]. These domains are highly related in that they are all composed of six antiparallel alpha helicies, but there are structural variations between domains within the family. For example the DD and the CARD rely on different helicies to

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facilitate interaction with other DD or CARD motifs. It has been shown that the CARD/CARD interactions of APAF-1 and procaspase 9 are mediated by helicies 2 and 3 of APAF-1, and helicies 1 and 4 of procaspase 9 which has been termed a type I interaction[7]. In contrast, DD/DD interactions between Pelle and Tube, two drosophila proteins important in degradation of the I κB homolog cactus rely on the loop regions between helices 1 and 2 and 4 and 5, as well as the loop between helices 5 and 6, which has been termed a type II interaction[8,9].

Death domains participate in homo and heterotypic interactions to promote downstream apoptotic signaling. The overall structure of death domains are similar however, the fold of the death domain varies and has been divided into two main types; subtype 1 and subtype 2. In subtype 1, helix 3 is antiparallel to helicies 2 and 4. In contrast, in subtype 2, helicies 2 and 4 are arranged parallel with one another, while helix 3 is nearly perpendicular to helicies 2 and 4.

DD-containing proteins propagate both pro-apoptotic and prosurvival signaling in response to ligand binding by binding to and recruiting other DD-containing proteins to multiprotein complexes. Signaling through death receptors requires an adaptor protein that is recruited to the receptor via its DD. The adaptor protein that is recruited to the receptor in response to Fas Ligand and TRAIL is FADD and recruitment of FADD to the Fas or TRAIL receptors (DR4 and DR5) is thought to be sufficient for the activation of caspase 8 and initiation of the apoptosis signal. FADD is also required for TNF dependent apoptosis, but another DD-containing protein, TRADD is recruited directly to the receptor. TRADD can then recruit either FADD to induce apoptosis or can recruit the DD- containing serine/threonine kinase Rip. Rip recruitment is required for Nuclear Factor κ B (NFκB) activation in response to TNF. As will be discussed later our ideas about these complexes have undergone recent revision and the situation now appears to be very complicated with different complexes in different locations having different effects. In addition, although we focus here on the activation of apoptosis through death domain interactions at death receptors, it should be remembered that death domain-containing proteins also have other effects. For example, the neurotrophin receptor p75 has a cytoplasmic death domain that resembles the TNFR1 death domain, but in contrast to TNFR1, Fas and the TRAIL receptors, apoptosis is activated not when the receptor binds to its ligand but instead when the ligand is withdrawn. The death domain of p75 has been classified into subtype 2 along with a number of other DD containing proteins including p100 NFκB, p105 NFκB, DAP kinase and myD88. Most of these proteins do not promote apoptosis upon overexpression and are often, though not always involved in inhibiting apoptosis. For example, both p100 and p105 are transcription factors of the Rel A family. Both proteins have a C-terminal DD, but only p100 has been implicated in the activation of apoptosis[10] and can activate a caspase 8-dependent apoptotic pathway and to sensitize cells to oncogenic RAS-induced apoptosis through as yet incompletely understood mechanisms.

The Fas death domain: The Fas death domain was the first death domain whose structure was solved using NMR and consists of six alpha helicies arranged antiparallel to one another[11]. Side chains of the hydrophobic residues form the core of the domain. Helicies 1 and 2 of the domain are located in the center while helicies 3 and 4 are on one side and 5 and 6 lie on the opposite side. When the structure was solved, the presence of a large number of charged residues on the surface of the domain suggested that electrostatic interactions mediate DD-DD interactions[11]. It was shown using deletion and mutagenic analysis that regions of the first cysteine rich domain of FAS led to disruption of receptor-receptor interaction and inhibited apoptotic induction in response to ligand[12]. Mutations in this region were also identified in patients with ALPS (Autoimmune lymphoproliferative syndrome) who are highly susceptible to lymphomas suggesting that Fas-mediated cell death achieved through interactions via these regions of the DD serves to remove the tumor cells. In addition, the role of FAS-induced death in the control of lymphocyte proliferation was identified by mutations in Fas in the lpr mouse[13]. This mouse strain serves as a model of human lupus as the mice develop similar symptoms with increasing age again suggesting that Fas signaling is important in controlling excess cell growth. In these mice a single point mutation (V238N) in the Fas DD is responsible for the phenotype. The V238N mutation greatly reduced self-association and binding to FADD compared to the wild-type protein. In addition, residues in the loop between helices 2 and 3 and within helix 3 blocked self-association.

The FADD death domain: The adaptor protein FADD is required for apoptotic signaling downstream of nearly all death receptors. The death domain of FADD is the most extensively studied of the DD-containing proteins. Like Fas, the FADD DD is composed of six alpha helicies arranged antiparallel to one another[14]. Again, helices 1 and 2 form the central portion of the domain, although in contrast to the Fas death domain, helices 3 and 6 are

located on one side while 4 and 5 are located on the other. Helix 6 is tightly packed against the interlocking helices while helix 3 is more loosely associated. Helix 2 is composed of primarily positively charged residues while helix 3 is largely negatively charged. Helix 1 and helix 6 are also negatively charged.

Extensive site directed mutagenesis has been performed on the FADD-DD. Initial characterization of important residues for mediating FADD-Fas interaction were charged residues localized primarily in helices 2 and 3. It was also suggested that the flexibility of helix 3 maximized contacts between interacting amino acids of helices 2 and 3[14]. However, more recent experiments have challenged these ideas and revealed that an expanded surface of the FADD-DD is actually important in binding to Fas. While the new data supports the previous view that helices 2 and 3 are required for the interaction, the new data revealed that residues in helices 1, 2, 3, 5 and 6 are also required for binding to Fas[15]. More surprisingly, in addition to the expansion of the binding surface to include more of the DD, data has recently been generated that indicates that residues within the DED of FADD (an entirely different domain of the protein) are also important in mediating the Fas-FADD interactions[16]. Using a modified reverse two-hybrid screen designed to identify random mutations in FADD that lost the ability to bind to Fas but retained binding to TRADD, it was found that mutations in residues that flank helix 5 of the DED abrogated Fas but not TRADD interaction. This established for the first time that the two domains do not function independently as was initially thought and that there is cooperativity in binding between the two domains.

FADD is also required for pro-apoptotic signaling in response to TNF Related Apoptosis Inducing Ligand (TRAIL). Again, FADD recruitment to the TRAIL receptors DR4 and DR5 was thought to be mediated by DD interactions as it has been shown that a dominant negative mutant of FADD that retains the DD but lacks the DED (DN-FADD) can be recruited to activated TRAIL receptors to inhibit TRAIL-induced death[17]. However, as is the case with Fas-FADD binding, more recent work has shown that regions in the DED are important for FADD-DR5 binding[18]. In fact the same DED-mutations that abrogated FADD-Fas binding also inhibit FADD-DR5 binding. Compensating mutations were then made to identify second site mutations that would restore binding and these mutations were also in the DED of FADD further establishing that the DED and the DD function in a cooperative manner to promote protein-protein interactions[18]. Together, these data show us that while the FADD DD is essential and, in the context of overexpression of the truncated DN-FADD molecule, sufficient for binding to the DDs of activated Fas or TRAIL receptors, the original view that the interaction occurs solely between the two death domains is not correct.

The TNFR1 death domain: The solution structure of the death domain of TNFR1 has been solved[19] in the context of a point mutant that inhibited self-association of the domain. Using TNFR1 R347A or R347K, which lies in helix 2, it has been shown that it too is composed of six alpha helicies and is structurally similar to the other death domains. However, the orientation of helicies 2, 3 and 4 are different than is found with Fas or FADD. While helices 2 and 4 are arranged parallel with one another, helix 3 is nearly perpendicular to helicies 2 and 4. In contrast, helix 3 is antiparallel to helicies 2 and 4 in the FADD and Fas death domains. The same orientation is seen in the neurotrophin receptor death domain. Other differences are also present. For example, helix 4 is longer in TNFR1 than in Fas or FADD, while helix 1 is considerably shorter than that of the other mammalian death domains. It has been shown the helices 2, 3 and 4 of TNFR1 are important for mediating both self-association and binding to TRADD[20]. Consistent with other death domains, data with the TNFR1 death domain indicated that homodimerization is inhibited at increasing salt concentrations supporting the idea that electrostatic interactions mediate self association as well as association with TRADD. In contrast to Fas death domain interactions where as discussed above, residues in all of the helicies mediate interactions, with the TNFR1 death domain helicies 2,3, and 4 are important in TNFR1-TRADD and TNFR1-TNFR1 interactions, but only the beginning of helix 3 is thought to be required for self-association and TRADD binding.

The TRADD death domain: The TNFR1 adaptor protein TRADD mediates signaling events downstream of the TNFR1 by binding to several death domain-containing proteins. Binding of TRADD to FADD or Rip promotes either apoptosis or NF□B activation in response to TNF. TRADD binds to FADD to promote caspase 8 dependent apoptosis. Based on our understanding of Fas signaling, this was originally thought to occur at the receptor- i.e. it was thought that the TNFR1 DD would bind TRADD (through its own DD) and that the TRADD DD would simultaneously interact with FADD through its DD. We now know that this idea is wrong. In fact the pro-apoptotic signaling events in response to TNFR1 stimulation occur in a cytoplasmic complex that contains TRADD, FADD and caspase-8 as well as RIP but is not associated with the receptor[21].

It has been suggested based on site directed mutagenesis studies that the binding surface required for FADD-Fas interaction is the same as that required for

FADD-TRADD binding[22]. These experiments suggested that helix 2 and helix 3 were key determinants of the interaction. However this work was performed with murine FADD and human TRADD and subtle differences between species could have caused important determinants of the interaction to be missed. Our understanding of FADD-TRADD binding is not nearly as comprehensive as that obtained studying FADD-Fas interactions and, unlike either Fas or TRAIL receptors there is as yet no evidence for a role of the FADD DED in the interaction with TRADD. Moreover, the structure of the TRADD death domain has been studied to a much lower resolution compared to the other death domain-containing proteins and all we really know is that as expected it is primarily alpha helical[23]. Alanine scanning mutagenesis of the death domain of TRADD has however been performed[24]. Through this work, it was suggested that the entire death domain probably mediates interaction with both the TNFR1 and FADD. It was not possible to subdivide the death domain into regions required for NFκB activation and regions required for induction of apoptosis suggesting that the interactions required for Rip and FADD binding may overlap[24]. To add to the complexity, because it is thought that a multimer of TRADD is required for interaction with the TNFR1, self-association of TRADD must also occur and the receptor bound complex probably involves simultaneous TRADD-TRADD, TRADD-TNFR1 and TRADD-RIP interactions while the pro-apoptotic cytoplasmic complex presumably involves combinations of TRADD-TRADD, TRADD-FADD and TRADD-RIP interactions. Since we do not know the stoichiometry of all these interactions some of them could be exclusive and some could occur simultaneously with other interactions.

Recent data from our lab adds an even greater level of complexity to the picture. Morgn *et al.*[25] found that TRADD is a nuclear shuttling protein suggesting that it might also bind to nuclear proteins. Moreover, we showed that an apoptotic pathway can activated by the accumulation of nuclear TRADD suggesting that these nuclear protein-protein interactions might promote apoptosis. Using a truncated protein that lacks the nuclear export signal but contains the DD and the nuclear localization signal, we showed that a TRADD molecule that is exclusively in the nucleus is a very effective inducer of apoptosis. However, the apoptotic pathway activated by expression of nuclear TRADD is not dependent upon interaction with FADD or activation of caspase 8 as is the case for death induced by the cytoplasmic TRADD-FADD-caspase-8 complex. Instead death induced by the nuclear protein involves the mitochondrial dependent apoptotic pathway and requires caspase 9 expression and catalytic activity (L.M.B. *et al.*, unpublished data). These data have led us to suggest that different protein-protein interactions mediate the cytoplasmic and nuclear apoptotic pathways activated by the adaptor protein TRADD. Thus our current picture of TRADD signaling is that TRADD can participate in at least three distinct complexes through its DD. In the first, which occurs at the cell membrane in association with the activated TNFR1 receptor, TRADD interacts with TNFR1, TRAF proteins and RIP and this leads primarily to anti-apoptotic signaling. After TNFR1 stimulation a second cytoplasmic complex involving TRADD interactions with FADD, caspase 8 and RIP leads to caspase 8-dependent apoptosis. Finally a third, nuclear complex that does not involve FADD or caspase 8 can also lead to apoptosis but this occurs through caspase 9.

Summary : Although it is now well established that death domain interactions are key regulators of apoptosis, there is still a long way to go before we completely understand how these interactions are regulated. The most extensively characterized of the death domain containing proteins in terms of protein-protein interactions is the FADD-DD. But as we learn more about the interactions of this protein the situation becomes more and more complicated. For example, studies initially characterized the important residues mediating FADD/Fas binding as lying in helicies 2 and 3 but recently the binding surface for has been extended to include regions within the DED as well as the loop between helicies 5 and 6 of the DD itself. These same regions have been shown to be important in binding to DR5 but different regions may be involved in binding to TRADD and TRADD itself can be involved in completely different apoptotic pathways that do not even involve FADD. Further structural and mutational analysis of these interactions will hopefully allow us to make better sense of these interactions and hopefully reach a stage where we can begin to consider adopting ways to modulate the interactions in a controlled way. When we reach this stage we hope to use this information design new therapeutic strategies to manipulate these responses. Since interactions between death domains are implicated in the development and treatment of cancer and other diseases, this could have important repercussions for human health.

ACKNOWLEDGMENTS

Work in the Thorburn lab has been supported by grants from NIH, US Army Breast and Prostate Cancer Research Programs and the Susan G. Komen breast cancer foundation.

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Molecular Biology of the Cell
Vol. 16, 1189–1199, March 2005

Selective Inactivation of a Fas-associated Death Domain Protein (FADD)-dependent Apoptosis and Autophagy Pathway in Immortal Epithelial Cells

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Submitted October 18, 2004; Accepted December 16, 2004
Monitoring Editor: Gerard Evan

Although evasion of apoptosis is thought to be required for the development of cancer, it is unclear which cell death pathways are evaded. We previously identified a novel epithelial cell death pathway that works in normal cells but is inactivated in tumor cells, implying that it may be targeted during tumor development. The pathway can be activated by the Fas-associated death domain (FADD) of the adaptor protein but is distinct from the known mechanism of FADD-induced apoptosis through caspase-8. Here, we show that a physiological signal (tumor necrosis factor-related apoptosis-inducing ligand) can kill normal epithelial cells through the endogenous FADD protein by using the novel FADD death domain pathway, which activates both apoptosis and autophagy. We also show that selective resistance to this pathway occurs when primary epithelial cells are immortalized and that this occurs through a mechanism that is independent of known events (telomerase activity, and loss of function of p53, Rb, INK4a, and ARF) that are associated with immortalization. These data identify a novel cell death pathway that combines apoptosis and autophagy and that is selectively inactivated at the earliest stages of epithelial cancer development.

INTRODUCTION

Because apoptosis can suppress tumor development, it is sometimes thought that cancer cells are generally resistant to apoptosis, whereas normal cells are sensitive. In fact, cancer cells are closer to their apoptotic threshold than their normal counterparts and often die more easily than normal cells in response to apoptotic stimuli (Evan and Vousden, 2001; Lowe *et al.*, 2004). Apoptosis sensitization in cancer cells occurs because growth-promoting oncogenic events such as Myc expression (Evan and Littlewood, 1998; Evan and Vousden, 2001; Pelengaris *et al.*, 2002), Rb inactivation (Chau and Wang, 2003), E2F activation (Nahle *et al.*, 2002), and cyclin D3 expression (Mendelsohn *et al.*, 2002) raise the levels of apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. Activated oncogenes can also sensitize cells to apoptosis by promoting loss of inhibitors of apoptosis that exist in primary cells (Duelli and Lazebnik, 2000). Immortalization and transformation also sensitize cells to nonapoptotic death (Fehrenbacher *et al.*, 2004).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-10-0906) on January 5, 2005.

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Abbreviations used: DD, death domain; FADD, Fas associated death domain protein; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

If cancer cells die more easily than their normal counterparts, which cell death pathways are evaded during tumor development? One answer is that cancer cells must remain below the lowered apoptotic threshold for undergoing stress-induced apoptosis that is caused by the oncogenes that drive cell growth. Indeed, it has been suggested that this may be sufficient to cause cancer without any other cellular defects (Green and Evan, 2002). However, this model does not exclude the possibility that there may also be specific cell death pathways that inhibit cancer development in normal cells that are specifically inactivated during tumor development. Such a pathway would be expected to have the unusual characteristics of working in normal cells but not in cancer cells, and signaling proteins and physiological stimuli that activate this kind of pathway should kill normal cells by mechanisms that are selectively inhibited during the transformation process without affecting other cell death pathways.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising treatment for cancer that kills tumor cells with little toxicity to normal tissues in preclinical models (LeBlanc and Ashkenazi, 2003). TRAIL induces apoptosis by binding to two receptors (DR4 and DR5) that contain an intracellular death domain (DD). Ligand binding is thought to result in conformational changes that expose a binding surface for the Fas-associated death domain (FADD) adaptor protein (Thomas *et al.*, 2004a,b). FADD also binds to procaspase-8, resulting in caspase-8 dimerization and activation (Boatright *et al.*, 2003; Boatright and Salvesen, 2003; Donepudi *et al.*, 2003), eventually leading to effector caspase activation. This well-established mechanism causes caspase-dependent apoptosis, which can be blocked by caspase inhibitors or a dominant negative version of FADD (FADD-DD also known as FADD-DN) that has an intact DD

but lacks the death effector domain and cannot bind procaspase-8.

We previously identified an alternate method by which FADD, through its DD alone can kill cells (Morgan *et al.*, 2001; Thorburn *et al.*, 2003). FADD-DD–induced death was unexpected because this molecule is a widely used inhibitor of apoptosis and is unusual because it occurs in primary normal epithelial cells but not in tumor cell lines and involves both the activation of caspases through caspase-9 (not caspase-8) and a separate activity that can be blocked by a serine protease inhibitor [4-(2-aminoethyl)benzenesulfonyl fluoride; AEBSF] (Thorburn *et al.*, 2003). Our previous studies raise several questions. Can a physiological stimulus activate the FADD-DD pathway or is it only induced by overexpression? What is the nature of the caspase-independent cell death that occurs in response to FADD-DD? And, when during epithelial cell transformation do cells lose the ability to respond to this pathway? Here, we answer these questions by showing that the FADD-DD pathway can be activated by a physiological signal (TRAIL receptor activation) working through the endogenous FADD protein and that when caspases are inhibited, the pathway does not kill by apoptosis but instead cells die by autophagy. We also identify a specific step in the transformation process (immortalization) when the pathway is selectively inactivated and show that this occurs via a mechanism that is separate from the known activities that occur during immortalization. These data identify a novel programmed cell death pathway involving apoptosis and autophagy that is selectively disrupted at the earliest stages of epithelial cell transformation.

MATERIALS AND METHODS

Cell Culture and Reagents

Isolation and culturing of normal human prostate epithelial cells from tissue samples was performed as described previously (Morgan *et al.*, 2001; Thorburn *et al.*, 2003). Human breast epithelial cells expressing defined transforming proteins were maintained as described previously (Elenbaas *et al.*, 2001). Tumor cell lines were obtained and cultured as recommended by American Type Culture Collection (Manassas, VA). The mouse mammary epithelial cells were cultured as described previously (Medina and Kittrell, 2000). Mice were obtained from the National Cancer Institute Mouse Models of Human Cancer Repository (Frederick, MD). Mammary tissue was isolated from 6- to 8-wk-old virgin mice, minced, and treated with 400 U/ml collagenase for 1.5–2 h and Pronase (1 U/ml) for 20 min. After digestion, epithelial cells were separated in a Percoll gradient and then cultured on collagen-coated plates in supplemented DMEM/F-12 medium with 1% fetal bovine serum. Recombinant human TRAIL was obtained from Calbiochem (San Diego, CA) and used at 100 ng/ml; zVAD.fmk was obtained from Alexis (San Diego, CA) and used at 0.1 mM. 3-methyladenine (3-MA), cycloheximide, and H33258 (bis-benzimide, no. 33258; Aventis, Strasbourg, France) were obtained from Sigma-Aldrich (St. Louis, MO) and used at 10 mM, 0.8 μg/ml, and 10 μg/ml, respectively. Antibodies for Western blotting experiments were obtained from Cell Signaling Technology (Beverly, MA).

Microinjection, Adenovirus Infection, and Cell Death Assays

Single cell-based microinjection experiments and cell death/survival assays were performed as described previously (Thorburn *et al.*, 2003). Fifty to 100 cells were injected for each plasmid in each experiment. Each injected cell was identified by virtue of its yellow fluorescent protein (YFP) fluorescence, and its fate was determined after incubation for 20 h. Because ~100 cells were injected at a time for each treatment, it was not possible to perform Western blotting to assess the expression level for YFP, YFP-FADD-DD, or the mutant proteins. Expression levels were therefore determined by visually assessing the amount of YFP fluorescence. The injected cell displayed similar levels of fluorescence, indicating that equivalent levels of each protein was compared. The percentage of living flat intact cells (rounded cells were scored as dead) was calculated for each experiment and the mean percentage of survival ± SD was calculated from at least four separate experiments by using different preparations of cells and plasmids. Survival >100% indicates that the cells grew during the experiment. Adenovirus purifications were performed using $CsCl_2$ centrifugation of doxycycline-regulated AdpEYFPc1, AdpEYFPc1-FADD-DD, and Tet repressor adenoviruses together with AdpEYFPc1-FADD-DD point mutant (V108E), which was constructed as described previously (Thorburn *et al.*, 2003). Cells were infected with ~20 plaque-forming units/cell of each virus for 4 h at which time the virus-containing medium was replaced with regular tissue culture medium. Adenoviral gene expression was repressed with 1 μg/ml doxycycline, and expression was induced by removing doxycycline. These conditions produced >90% infection efficiency as determined by YFP fluorescence. Population-based cell viability assays after adenovirus infection and treatment with TRAIL, and protease inhibitors were performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay from Promega (Madison, WI) as described in the manufacturer's instructions. Time-lapse microscopy was performed in an environmental chamber attached to a Zeiss Axiovert S200 microscope by using a 32× objective. Images were captured at 15-min intervals by using a Hamamatsu charge-coupled device (Malvern, PA) camera run by Openlab (Improvision, Warwick, United Kingdom) software, saved in QuickTime Movie format, and frames were captured for still images.

Autophagy Assays

For electron microscopy, cells were cultured in 6-cm dishes, treated with TRAIL or infected with FADD-DD–expressing adenoviruses in the presence of the caspase inhibitor zVAD.fmk as indicated on the figure legends and incubated overnight. Cells were fixed with 2.5% phosphate-buffered gluteraldehyde, postfixed in 1% phosphate-buffered osmium tetroxide, embedded in Spurr's resin, sectioned, double stained with uranyl acetate and lead citrate, and analyzed using a Philips 400 transmission electron microscope. For each treatment and control group, 20–50 randomly chosen cells were analyzed for morphological features associated with autophagy. Cells were scored as autophagy positive by using a scoring method described by Yu *et al.* (2004), where cells with <10 vesicles/cell were scored as normal, 10–19 vesicles/cell were scored as mild autophagy, 20–29 vesicles/cell were scored as moderate autophagy, and >30 vesicles/cell were scored as severe autophagy. The histograms show the percentage of cells in each category. The percentage of the total cell area taken up by autophagic vesicles for each randomly chosen cell was determined using Adobe Photoshop software. For analysis of green fluorescent protein (GFP)-LC3 localization, cells were injected with the expression plasmid along with FADD-DD or control expression plasmids, and time-lapse fluorescence microscopy was performed. Still images were captured from the movies.

RESULTS

A Physiological Stimulus Can Activate the FADD-DD Pathway through the Endogenous FADD Protein

We previously made the surprising discovery that the death domain of FADD can kill normal epithelial cells (Morgan *et al.*, 2001) and showed that FADD-DD–induced cell death involves both caspases and an activity that can be inhibited by AEBSF (Thorburn *et al.*, 2003) that cause different morphological phenotypes in the dying cells. To determine whether a physiological stimulus working through the endogenous FADD protein also could activate this pathway, we examined TRAIL receptor signaling. We reasoned that if TRAIL can activate the FADD-DD–dependent pathway, TRAIL-induced death of normal cells should be inhibited only when caspases and serine proteases are blocked simultaneously. In contrast, caspase inhibitors such as zVAD.fmk alone should block TRAIL-induced death in cancer cells.

For these experiments, we treated normal primary human prostate cells (sensitive to FADD-DD) or DU145 prostate cancer cells (insensitive to FADD-DD) with recombinant TRAIL in the presence of low doses of cycloheximide, which inhibited protein synthesis by ~70% (our unpublished data) and was unable to induce cell death by itself (Figure 1A). Cycloheximide treatment was required in both the normal and cancer cells to allow TRAIL-induced cell death. Cell death was monitored by time-lapse microscopy after treatment with zVAD.fmk and AEBSF. TRAIL killed both cell types, and in both cases the morphology of the dying cells was consistent with apoptosis. However, although zVAD-.fmk alone was able to block cell death in cancer cells, only the combination of zVAD.fmk and AEBSF could inhibit cell death in normal cells. The caspase inhibitor on its own did,

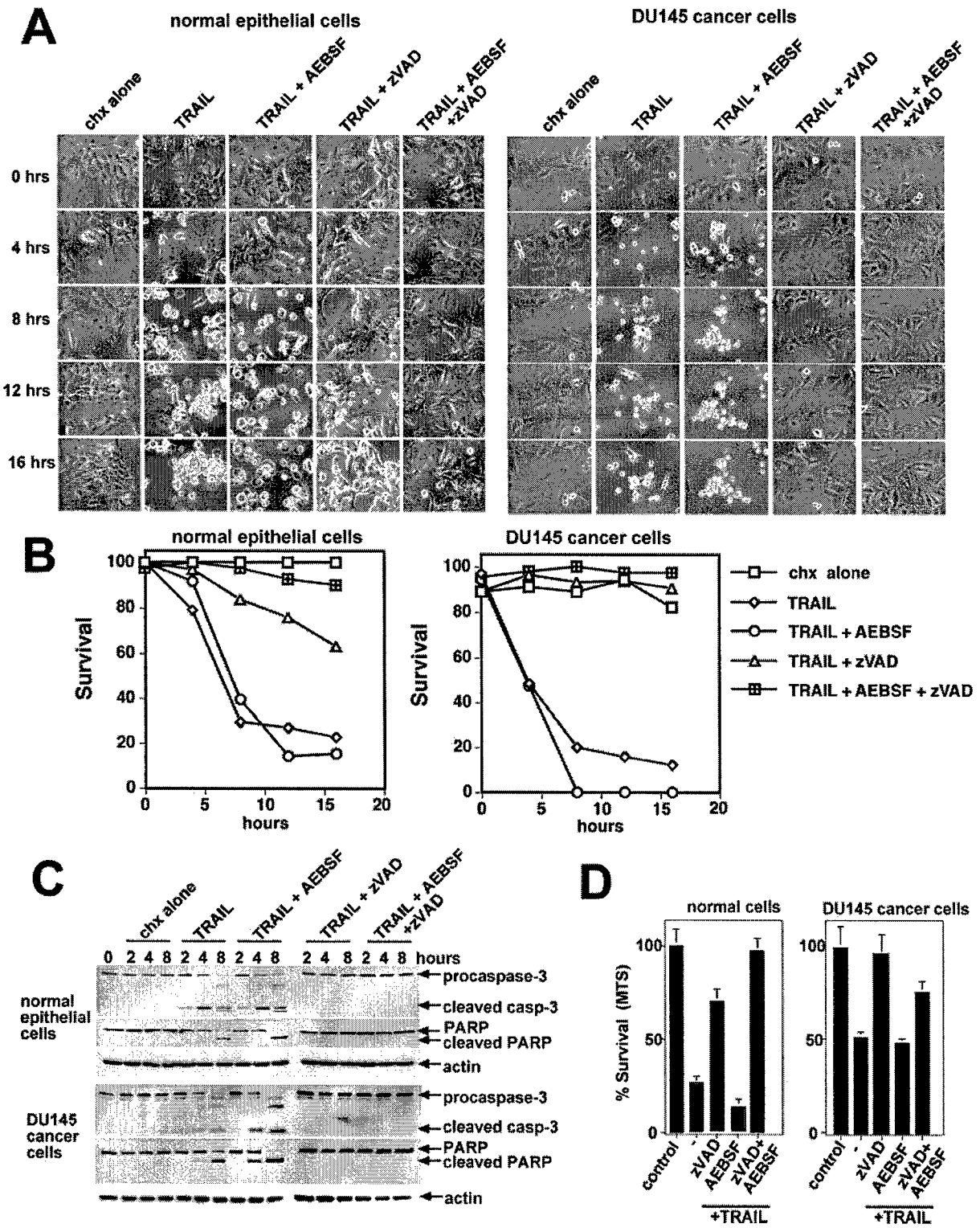

Figure 1. TRAIL can kill normal and cancerous epithelial cells by different mechanisms. (A) Time-lapse microscopy of normal prostate epithelial cells or prostate cancer cells treated with TRAIL plus AEBSF and zVAD.fmk. TRAIL kills both cell types, but zVAD.fmk alone can protect only the cancer cells; the combination of zVAD.fmk and AEBSF is required to protect normal cells from TRAIL-induced death. (B) The number of dead cells for each time point was determined by counting rounded cells in individual frames for each treatment. Treatment with zVAD.fmk in the normal cells altered the slope of the line, indicating that the caspase-independent cell death response in normal cells occurred more slowly than caspase-dependent cell death. (C) Normal or cancerous prostate cells were treated with TRAIL in the presence of the protease inhibitors and harvested for Western blot analysis of caspase-3 and PARP cleavage. In both cell types, caspase-3 was activated, leading to PARP cleavage, and the caspase inhibitor zVAD.fmk completely blocked the response. (D) MTS assays of TRAIL treated cells were performed. ZVAD.fmk only partially protected normal cells but completely protected cancer cells. The combination of zVAD.fmk and AEBSF completely protected normal cells.

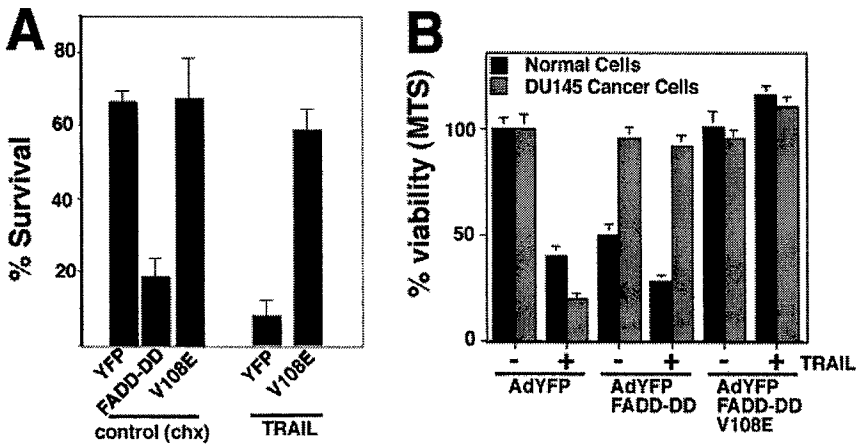

Figure 2. TRAIL activates the FADD-DD pathway through the endogenous FADD protein. (A) Normal prostate epithelial cells were injected with YFP control, FADD-DD, or FADD-DD V108E expression constructs in the presence or absence of TRAIL as indicated, and cell death was determined by monitoring the response of each injected cell. In the absence of TRAIL, FADD-DD induced apoptosis but the V108E mutant did not. The V108E mutant blocked TRAIL-induced cell death. (B) Normal cells or cancer cells were infected with doxycycline-regulated adenoviruses expressing YFP, FADD-DD, or FADD-DD V108E as indicated and then treated with or without TRAIL. Cell survival was determined using an MTS assay. TRAIL killed both normal and cancer cells, and in the absence of TRAIL, FADD-DD could kill only normal cells. In cancer cells, both the V108E mutant and the wild-type FADD-DD were equally effective at inhibiting TRAIL-induced cell death. In the normal cells, wild-type FADD-DD plus TRAIL led to increased cell death compared with either FADD-DD or TRAIL alone, whereas the V108E mutant completely inhibited TRAIL-induced cell death. These data indicate that FADD-DD functions differently in normal and cancerous prostate cells and can cooperate with TRAIL to increase normal cell death.

however, alter the morphology of the normal cells as they died in response to TRAIL. High doses of zVAD.fmk have nonspecific effects such as inhibition of cathespin B (Schotte *et al.*, 1999) that have been implicated in apoptosis regulation. Therefore, our data indicate that inhibition of either caspases or such nonspecific targets is sufficient to prevent death of the normal epithelial cells. Inhibition of caspases resulted in cell death that was associated with cell rounding and detachment but little if any membrane blebbing or cellular fragmentation. Quantitation of the number of dying cells for each treatment during the time course of the experiment (Figure 1B) confirmed that zVAD.fmk alone was able to prevent cancer cell death but had only a partial effect in normal cells. Interestingly, the partial inhibition of normal cell death by the caspase inhibitor also displayed different kinetics as demonstrated by the reduced slope of the line in the time course. These data suggest that the preferred mode of death in the normal cells is via caspase-dependent apoptosis and that the cell death that occurs when caspases are inhibited is slower. This conclusion is also supported by the fact that in the absence of inhibitors the morphology of both normal and cancer cells dying in response to TRAIL is consistent with classical apoptosis with membrane blebbing, cell contraction, and fragmentation. To confirm that caspases were inhibited in both cell types by zVAD.fmk, we assessed the processing of caspase-3 and its substrate poly(ADP-ribose) polymerase (PARP) (Figure 1C). In both normal and cancer cells, the caspase inhibitor completely blocked caspase-dependent cleavage, whereas AEBSF had no effect. Similar results were obtained using MTS assays for viability in normal cells or cancer cells treated with TRAIL (Figure 1D). Together, these data indicate that under these conditions, TRAIL can kill normal cells and cancer cells by different mechanisms with normal cells displaying caspase-independent cell death in addition to caspase-dependent effects. In contrast, and in agreement with a large number of published studies in various cancer cell lines, cancer cells die by caspase-dependent apoptosis in response to TRAIL.

If TRAIL can activate the FADD-DD pathway through the endogenous FADD protein, an FADD-DD mutant that cannot cause cell death when it is expressed in normal cells should function as a dominant negative inhibitor of TRAIL-induced apoptosis. Such a molecule will also be unable to activate caspase-8 because it lacks the DED and blocks death in cancer cells, too. In contrast, the wild-type FADD-DD molecule should cooperate with TRAIL to increase normal cell death through the FADD-DD pathway but inhibit TRAIL-induced cancer cell death because this should occur only through the established caspase-8–dependent pathway. We tested several point mutants and identified a mutant (V108E) that is unable to induce normal epithelial cell death when injected into cells on its own but is able to bind to TRAIL receptors (Thomas *et al.*, 2004a) and can block TRAIL-induced cell death (Figure 2A).

We expressed wild-type FADD-DD or the V108E mutant in a population of cells from a doxycycline-regulated adenovirus and then treated the normal and cancer cells with TRAIL and measured the response by using a population-based cell viability assay. Figure 2B shows that wild-type FADD-DD increased TRAIL-induced death in normal epithelial cells but blocked TRAIL-induced death in cancer cells. In contrast, the V108E mutant blocked TRAIL-induced death in both normal cells and cancer cells. These data suggest that TRAIL can work through the FADD-DD pathway in normal epithelial cells and that this pathway cannot be activated in cancer cells where all TRAIL-induced cell death occurs through the established caspase-8 pathway.

FADD-DD and TRAIL Can Induce Autophagy in Normal Epithelial Cells

Autophagy has been implicated in tumor suppression (Edinger and Thompson, 2003; Qu *et al.*, 2003; Yue *et al.*, 2003; Alva *et al.*, 2004; Gozuacik and Kimchi, 2004) and has been linked to TRAIL-induced epithelial cell death (Mills *et al.*, 2004). We therefore tested whether autophagy occurs in FADD-DD–expressing normal epithelial cells by using transmission electron microscopy (TEM). Normal epithelial cells expressing adenoviral FADD-DD had numerous membrane-bound vesicles often containing organelles and other cellular fragments (Figure 3). In contrast no significant increase in vesicles was found when the cells expressed the V108E FADD-DD mutant that is unable to kill. Similar vesicle formation occurred in normal cells after treatment with TRAIL. Vesicle formation, which is characteristic of autophagy (Gozuacik and Kimchi, 2004), occurred in the presence of the caspase inhibitor zVAD.fmk, indicating that it is separate from the caspase-dependent apoptosis that occurs in

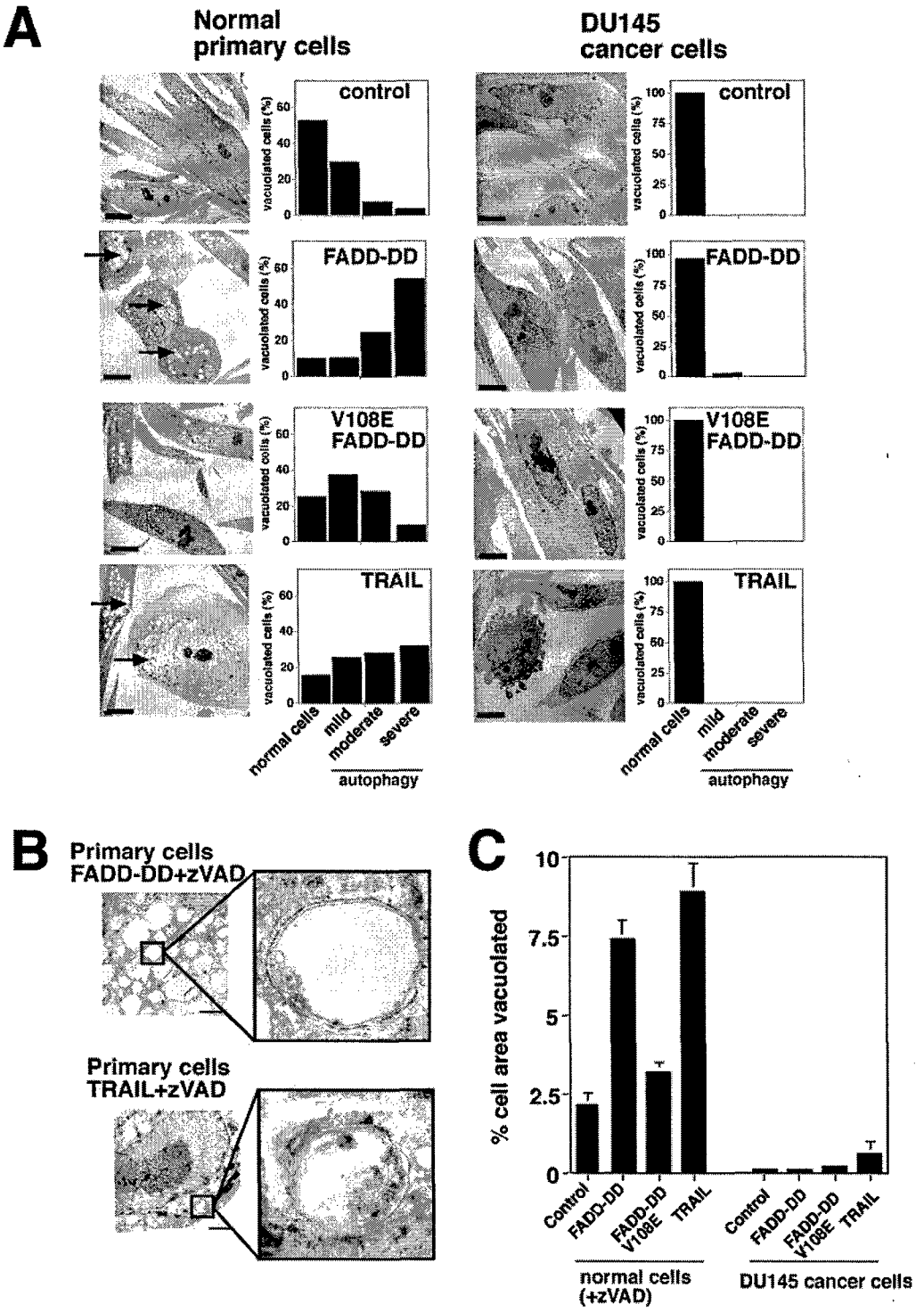

Figure 3. FADD-DD can cause autophagic vesicle formation in normal epithelial cells. (A) Normal primary prostate epithelial cells or DU145 prostate cancer cells were treated with TRAIL or infected with adenoviruses expressing FADD-DD or V108E FADD-DD as indicated and analyzed by TEM. Large numbers of vesicular structures (arrows) were found in normal cells expressing FADD-DD or treated with TRAIL. Normal cells were treated with zVAD.fmk to prevent caspase-dependent signaling from obscuring any caspase-independent effects. Bars, 5 μm. (B), higher power images of autophagic vesicles from FADD-DD or TRAIL-treated normal prostate cells showing double membranes and cellular debris. Bar, 0.5 μm. (C) Cell area taken up by autophagic vesicles, indicating that FADD-DD and TRAIL increase the proportion of each normal cell that is vacuolated.

the normal cells expressing FADD-DD or treated with TRAIL and implying that it participates in the caspase-independent arm of the cell death pathway that is induced by FADD-DD. Consistent with this idea, vesicles were not formed in response to FADD-DD or TRAIL in prostate cancer cells.

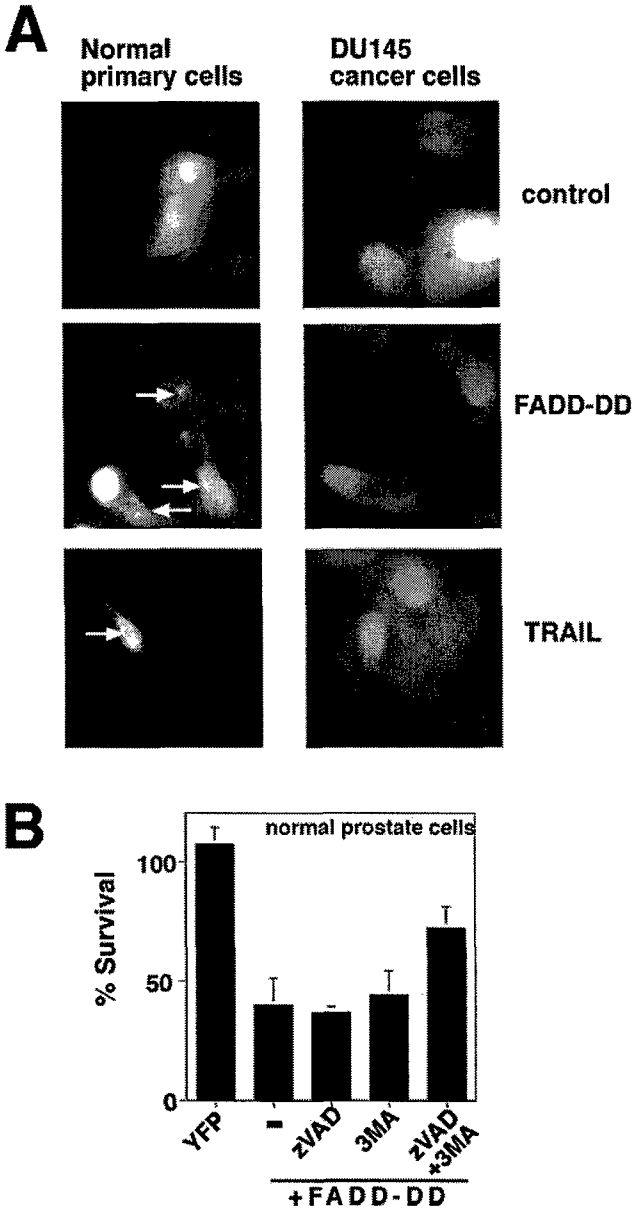

Figure 4. FADD-DD-induced autophagy in normal cells. (A) Normal prostate cells or DU145 cancer cells were injected with GFP-tagged LC3 plus FADD-DD or treated with TRAIL and followed by fluorescence microscopy. GFP-LC3 forms aggregates (arrows) in FADD-DD–expressing or TRAIL-treated normal cells but does not aggregate in cancer cells. (B) Normal prostate cell survival 24 h after injection with FADD-DD and treatment with zVAD.fmk or 3-MA alone or in combination. FADD-DD–induced cell death is not prevented by either inhibitor alone but is inhibited by the combined inhibitors.

Another characteristic of autophagy is the translocation of LC3 to autophagic vesicles, which can be detected as aggregates of GFP-tagged LC3 (Kabeya *et al.*, 2000). We therefore injected normal primary epithelial cells or cancer cells with untagged FADD-DD or V108E expression vectors along with a GFP-tagged LC3 protein. The aggregation of GFP-LC3 into dots was assessed by fluorescence microscopy (Figure 4A). Aggregation of LC3 occurred in response to FADD-DD in normal cells but not in cancer cells, aggregation started before any morphological signs of cell death were apparent, and aggregation was not affected by the caspase inhibitor zVAD.fmk (our unpublished data). TRAIL treatment of the cells in the presence of zVAD.fmk had a similar effect. To test whether autophagy contributes to the FADD-DD and TRAIL-induced death that occurs in normal cells, we asked whether the autophagy inhibitor 3-MA could block cell death either on its own or in combination with zVAD.fmk. 3-MA was unable to prevent FADD-DD–induced cell death on its own but did prevent cell death when combined with zVAD.fmk (Figure 4B). These data indicate that autophagy is involved in the caspase-independent cell death response to the FADD-DD signaling pathway in normal epithelial cells.

Selective Disruption of FADD Death Domain-induced Cell Death Occurs When Epithelial Cells Are Immortalized

A distinctive feature of the FADD-DD cell death pathway is that it works in normal prostate epithelial cells but does not work in cancer cells. This raises the question of whether other epithelial cell types behave similarly and, more importantly, when during the transformation process resistance to this pathway arises. To address these questions, we examined human breast epithelial cells that were immortalized and transformed by defined genetic changes (expression of the telomerase catalytic subunit (TERT), SV40 Large T and small t antigens, and oncogenic Ras) (Elenbaas *et al.*, 2001). The cells were derived by expressing the transforming proteins in normal primary human mammary epithelial cells (HMECs) and thus represent a set of cells at different steps in the transformation process arising through defined genetic changes. FADD-DD was expressed in each set of cells by microinjection, and cell death was determined by following the fate of each FADD-DD–expressing cell. Figure 5A shows that normal HMECs and the TERT-expressing HME cells were sensitive to FADD-DD–induced cell death; however, HMECs expressing TERT plus SV40 Large T antigen (HMLcE), Large T and small t antigens (HML), TERT, Large T and small t (HMLE), and cells expressing TERT, Large T, small t, and active Ras (HMLPR) were all resistant to FADD-DD–induced death.

We next asked whether this resistance to cell death was specific to the FADD-DD–induced pathway by comparing the ability of FADD-DD, which cannot activate the caspase-8 pathway, and a full-length FADD protein that can bind caspase-8, to kill HME and HMLcE cells. A general apoptosis resistance mechanism arising in the immortal HMLcE cells should inhibit both FADD proteins. In contrast, a mechanism that selectively disrupts the FADD-DD pathway in HMLcE cells should not alter cell death in response to the FADD molecule that can activate caspase-8. HMLcE cells were resistant to FADD-DD, whereas both HME and HMLcE cells were killed equally well by full-length FADD (Figure 5B). These data indicate that selective resistance to FADD-DD–induced killing arises at a specific step during transformation and can be conferred by a viral oncogene (SV40 Large T antigen). These data also show that the FADD-DD pathway is not affected by TERT expression.

The TERT and T antigen-expressing HMECs are immortal but not transformed (Elenbaas *et al.*, 2001), suggesting that resistance to FADD-DD–induced cell death is associated with immortalization rather than transformation. We therefore tested whether spontaneously immortalized epithelial cells are resistant to FADD-DD. Because human cells very rarely undergo spontaneous immortalization, we used mouse epithelial cells and compared the response to FADD-DD in primary low passage cells to cells that had undergone spontaneous immortalization after continued culture. We also compared the re-

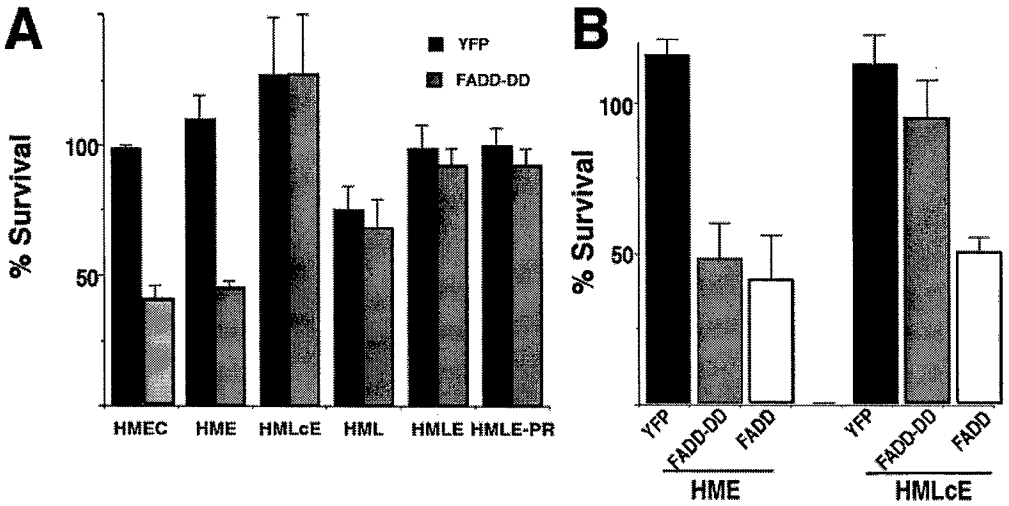

Figure 5. FADD-DD–induced cell death is selectively inhibited in immortalized cells. (A) HMECs at different stages of immortalization and transformation were injected with YFP control or YFP-FADD-DD expression vectors, and the percentage of survival for fluorescent cells was determined. FADD-DD killed normal HMECs and TERT-expressing HME cells, but it did not kill HMEC expressing T antigen plus TERT (HMLcE) or any of the other cells. Panel B, HME cells and HMLcE cells were injected with control, FADD-DD, or a full-length FADD construct that can activate caspase-8. Both FADD-DD and FADD could kill HME cells, but only the FADD molecule capable of activating caspase-8 killed HMLcE cells. These data show that resistance to FADD-DD–induced cell death arises in response to expression of T antigen, which causes immortalization and that this resistance is specific to the FADD-DD pathway.

sponse in primary fibroblasts from the same tissue pieces to test whether the response was epithelial specific. Figure 6A shows that primary mouse mammary epithelial cells (MMECs) were killed by FADD-DD, whereas spontaneously immortalized epithelial cells and primary nonimmortalized fibroblasts were resistant. All the cells underwent apoptosis in response to the full-length FADD protein that can activate caspase-8, indicating that the immortalized cells acquire selective resistance to the FADD-DD pathway rather than a general resistance to all apoptotic stimuli. Similar results were obtained in mouse prostate epithelial cells (our unpublished data). Together with our previous studies (Morgan *et al.*, 2001; Thorburn *et al.*, 2003), these data indicate that human and mouse prostate and breast epithelial cells respond to FADD-DD in the same way.

There are differences in the requirements for immortalization between cell types and between mouse and human cells (Romanov *et al.*, 2001; Drayton and Peters, 2002; Rangarajan and Weinberg, 2003). However, in all cells, it is thought that disruption of p53, INK4a/ARF (these two gene products from the same locus regulate the Rb and p53 pathways), and Rb are important steps in the immortalization process (Drayton and Peters, 2002; Hahn and Weinberg, 2002; Rangarajan and Weinberg, 2003). TAg inactivates p53 and Rb (Ali and DeCaprio, 2001). Disruption of the p53 pathway might therefore provide a simple explanation for the inability of FADD-DD to kill immortal tumor cells. We therefore examined the response to FADD-DD and a FADD molecule that can activate caspase-8 in MMECs from p53 knockout animals. Figure 6B shows that low passage primary epithelial cells from the p53 knockout animals were killed in response to FADD-DD, indicating that loss of p53 function does not affect the FADD-DD pathway and excluding this explanation for the immortalization-dependent resistance. As expected the p53−/− cells did not become senescent and grew well in culture. However, upon continued culture, the cells became resistant to FADD-DD–induced apoptosis but were equally sensitive to apoptosis induced by a FADD molecule that can bind and activate caspase-8. We next cultured epithelial cells from mice with knockouts of the p53 target gene p21, which controls cell cycle progression, and INK4a/ARF (both genes are inactivated in these animals, which have a deletion of exons 2 and 3; Serrano *et al.*, 1996). In each case, primary MMECs underwent FADD-DD–dependent apoptosis (Figure 6C). As with the p53 −/− cells, MMECs that lack functional INK4A/ARF genes became resistant to FADD-DD–induced apoptosis when they were continuously cultured (Figure 5D), suggesting that acquisition of selective resistance to this pathway confers an advantage to the cells.

SV40 T antigen also inactivates Rb, and we next asked whether this was responsible for resistance to FADD-DD. Because Rb knockout results in embryonic lethality (Jacks *et al.*, 1992), we isolated MMECs from animals with homozygous "floxed" Rb genes. These cells were infected with an adenovirus that expresses Cre recombinase to knockout the Rb gene. Three days after infection, there was no detectable Rb protein in the cells (Figure 6C, inset). FADD-DD injection into Rb-deficient cells resulted in apoptosis induction that was equally efficient as that observed with the FADD molecule that can activate caspase-8 (Figure 6C). Together, these data indicate that the FADD-DD pathway is selectively disrupted upon immortalization but that resistance does not arise as a result of the inactivation of p53, INK4a, ARF, p21, or Rb that occurs during immortalization.

Autophagy Is Inactivated in FADD-DD-resistant Epithelial Cells

If autophagy is involved in the FADD-DD pathway, it should occur in early passage mouse breast cells that express FADD-DD and should not be inhibited by zVAD.fmk. However, when cells acquire resistance to FADD-DD–induced cell death, they should also fail to show signs of autophagy. We therefore assessed autophagic vesicle formation in response to FADD-DD in low (passage 4, i.e., sensitive to FADD-DD–induced cell death) and high (passage 30, i.e., insensitive to FADD-DD–induced death) passage MMECs from INK4a/ARF knockout animals. These cells were chosen because they come from the same primary cell prepara-

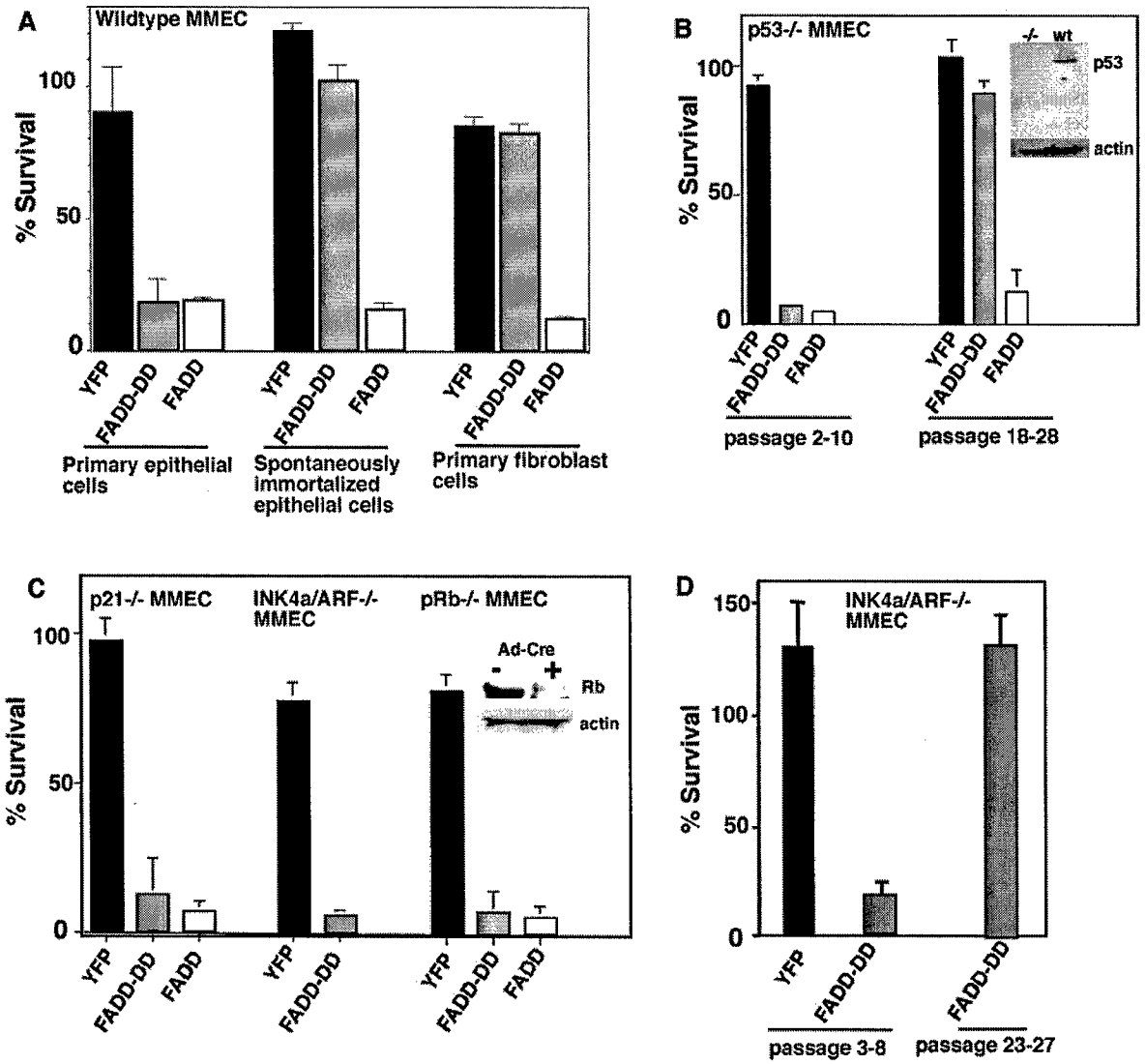

Figure 6. Inhibition of the FADD-DD pathway in immortal cells is not caused by inactivation of genes that are known to regulate immortalization. (A) Primary mouse mammary epithelial cells, spontaneously immortalized epithelial cells, or primary breast fibroblasts were injected with YFP control, FADD-DD, or FADD expression constructs, and cell survival was determined. All the cell types were killed by the FADD molecule that can activate caspase-8, but only the primary epithelial cells were killed by FADD-DD. (B) Mammary epithelial cells were isolated from p53 knockout mice and tested for sensitivity to FADD-DD after limited culture (passage 2–10) or extended in vitro culture (passage 18–28). FADD-DD killed the low passage number cells but could not kill the high passage number cells. The Western blot insert compares protein samples from the p53 knockout or wild-type animals showing that the cells lacked p53. (C) Primary MMECs were cultured from p21 and INK4a/ARF knockout animals or from animals with floxed Rb genes, which were subsequently infected with a Cre recombinase adenovirus and maintained for 3 d in culture at which time no detectable Rb protein was present (inset). All the low passage (< passage 10) primary cells underwent apoptosis in response to FADD-DD. (D) Low passage (passage 3–8) or high passage (passage 23–27) MMECs from INK4a/ARF −/− mice were injected with FADD-DD as indicated. Only the low passage cells were killed by FADD-DD.

tion and both grow well in culture yet they differ in their ability to die in response to FADD-DD expression. In addition, because the cells lack INK4a and ARF, both the Rb and p53 pathways are inactivated, thus removing potential confounding influences of other cell death pathways. The cells were infected with the FADD-DD- or V108E FADD-DD–expressing adenoviruses, treated with zVAD.fmk to block caspase activation, and assessed by TEM. Low passage cells expressing FADD-DD displayed high levels of autophagic vesicle formation; however, vesicles were not formed in low passage cells expressing the V108E mutant or in high passage cells expressing either wild-type or mutant FADD-DD (Figure 7).

DISCUSSION

In this article, we present data showing that the death domain of FADD can activate a cell death pathway involving both apoptosis and autophagy that is selectively inactivated when normal epithelial cells are immortalized. The same pathway can be activated by TRAIL receptor stimulation and blocked by a FADD-DD mutant, indicating that although the pathway is conveniently activated by expression of exogenous FADD-DD, a physiological signal also can activate this pathway through the endogenous FADD protein. Prostate and breast epithelial cells behave similarly but fibroblasts are unable to activate this pathway. Resistance to

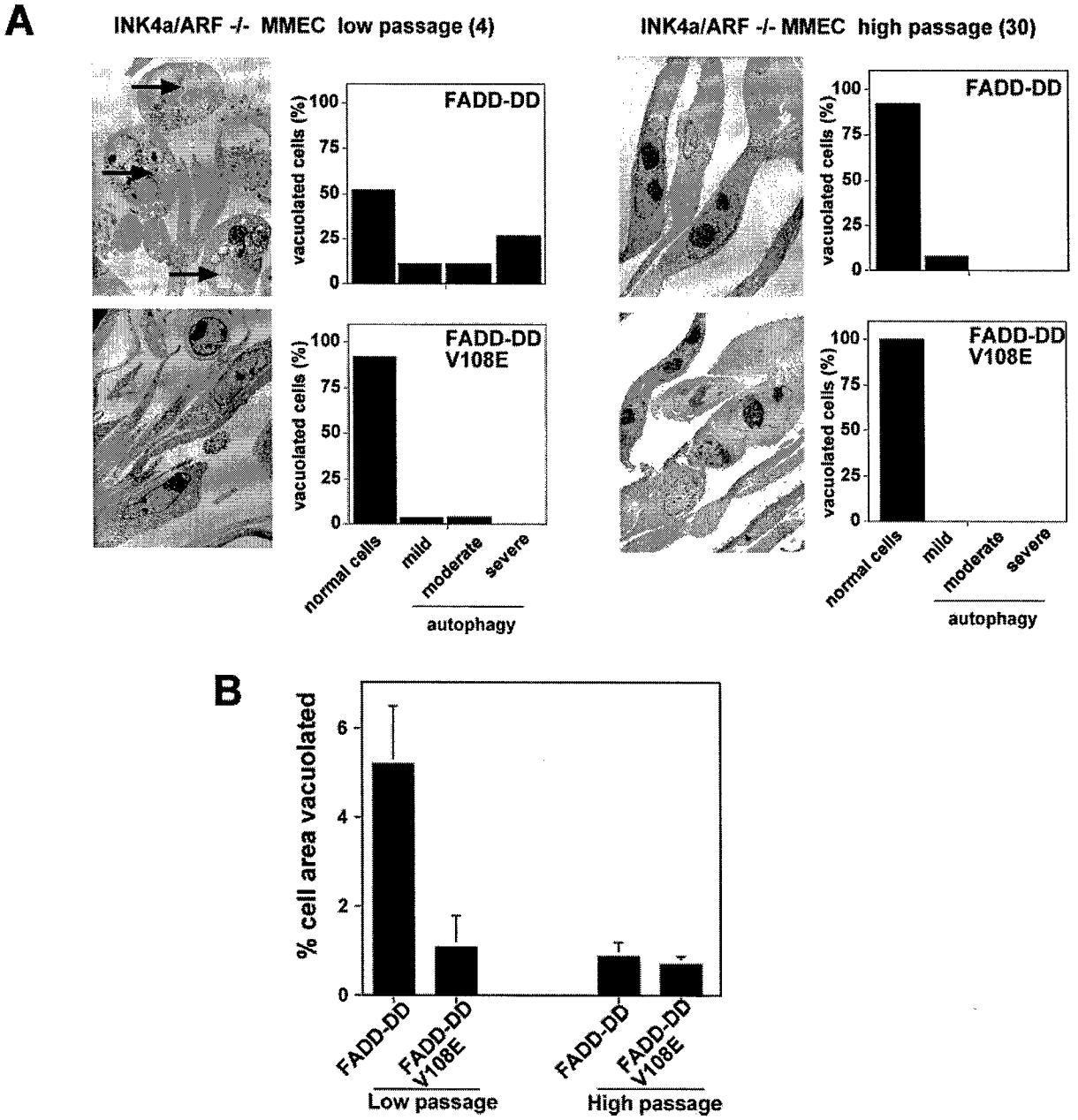

Figure 7. FADD-DD-induced autophagy is lost in late passage epithelial cells. (A) Low or high passage MMECs from INK4a/ARF knockout animals were infected with FADD-DD or V108E FADD-DD adenoviruses in the presence of zVAD.fmk to inhibit caspase-dependent effects and analyzed by TEM for signs of autophagy. Large numbers of vesicles (arrows) were observed only in the low passage cells expressing wild-type FADD-DD. (B) Cell area taken up by vacuolated structures, indicating that FADD-DD but not the V108E mutant causes an increase in such structures in low passage cells.

this form of cell death occurs without affecting other apoptosis pathways, including those that are induced by an FADD protein that can interact with and activate caspase-8 through a different part of the protein. This resistance arises at immortalization rather than complete transformation, suggesting that it represents an early cell death defect that occurs during the development of epithelial cancers. This work provides the first example that we are aware of where apoptosis and autophagy are induced in a cell type-specific manner and selectively disrupted during immortalization and transformation.

Our experiments demonstrating inhibition of TRAIL-induced death by the V108E mutant and cooperation between TRAIL and wild-type FADD-DD to increase killing of normal cells suggest that a stimulus that works through FADD can activate the FADD-DD apoptosis/autophagy pathway. It is important to note that this does not necessarily mean that the normal physiological stimulus is actually TRAIL or that the FADD-DD pathway is an important aspect of TRAIL signaling under normal circumstances. Moreover, because activation of the FADD-DD pathway by TRAIL in normal cells was only detected when we blocked the canonical

caspase-8–dependent pathway, the FADD-DD pathway may not be the major TRAIL-induced cell death pathway even if TRAIL is the physiological stimulus. Instead, it is possible that a different stimulus (perhaps not even involving death receptors) activates FADD to induce the FADD-DD pathway under normal circumstances. Because we have identified a point mutant (V108E) that cannot activate the FADD-DD pathway, it may be feasible to address these issues by creating a knockin mouse that contains the V108E mutation (which, if our ideas are correct, may have a cancer-related phenotype) and asking whether TRAIL signaling occurs properly in these animals.

We have not detected FADD cleavage in normal cells after treatment with TRAIL. In addition, we previously found that overexpression of a full-length FADD molecule containing a point mutation in the DED that prevents caspase-8 binding or expression of wild-type FADD in the presence of a caspase-8 inhibitor could kill normal but not cancerous cells (Thorburn *et al.*, 2003). We therefore do not suggest that the isolated FADD-DD protein occurs under physiological conditions or that the FADD-DD pathway is activated only by the truncated protein. Instead, we think that the FADD-DD pathway is activated by full-length FADD but that this is only evident when the canonical caspase-8 pathway is blocked. We therefore view the expression of the truncated FADD-DD protein, which provides the most effective way to activate this pathway without activating the canonical caspase-8 pathway, as a useful tool to selectively activate and study the pathway that is normally activated by the endogenous full-length FADD protein.

There are other recent examples where autophagy and apoptosis is combined. TRAIL-induced autophagy occurs during breast epithelial cell death to form acini in three-dimensional cultures (Mills *et al.*, 2004). However, this cell death, which occurred in immortal MCF10A cells, was blocked by FADD-DD, suggesting that it has some differences from the FADD-DD–induced death in nonimmortalized cells. In addition, DAP kinase, which has been implicated in death receptor-induced cell death (Cohen *et al.*, 1999), can cause autophagy in addition to apoptosis (Inbal *et al.*, 2002). Beclin 1, which promotes autophagy, is a haploinsufficient tumor suppressor (Qu *et al.*, 2003; Yue *et al.*, 2003) that displays reduced expression in breast tumors (Liang *et al.*, 1999), providing a genetic link between defects in autophagy and cancer development. Our work suggests that at least some such defects arise at the earliest steps in epithelial cancer development (i.e., the acquisition of immortalization) to inactivate specific cell death pathways that involve both caspase-dependent apoptosis and autophagy.

Although there are differences in the requirements for immortalization and transformation of human and mouse cells (Drayton and Peters, 2002; Rangarajan and Weinberg, 2003), mammary epithelial cells from both organisms behave identically in regards to FADD-DD–induced apoptosis/autophagy and are inhibited by immortalization in both cases. Prostate epithelial cells also behave the same way. Although immortalization is associated with acquired resistance to this cell death pathway, the known activities that are involved in mammalian cell immortalization, including telomerase activation, or loss of function of p53, INK4a, ARF, and pRb are not responsible for resistance to this cell death pathway. In addition, MMECs lacking p53, or INK4a and ARF, which do not undergo crisis or become senescent, become selectively resistant to the FADD-DD pathway upon continued culture. These data suggest that the acquisition of resistance to FADD-DD–induced cell death represents an uncharacterized aspect of immortalization that confers a selective advantage to the cells.

Although evasion of apoptosis is widely regarded as a hallmark of cancer (Hanahan and Weinberg, 2000), the cell death pathways that must be avoided are poorly understood. Because growth-promoting oncogenic events such as Myc expression or Rb inactivation sensitize cells to diverse apoptotic stimuli and function as an intrinsic tumor suppression mechanism (Lowe *et al.*, 2004), cancer cells must overcome this hurdle to remain below their apoptotic threshold. This can be achieved by altering components of the cell death machinery such as p53, ARF, or Bcl-2 family members that control diverse apoptotic pathways (Lowe *et al.*, 2004). The apoptosis/autophagy pathway that is induced by FADD-DD and TRAIL has unusual characteristics (normal epithelial cell specificity, inactivation when cells are immortalized without affecting other cell death pathways, and no inhibition by loss of p53 or ARF or Bcl-2 expression) that are unlike oncogenic sensitization to apoptosis and suggest it represents a specific hurdle that some cells must also overcome if they are to become cancerous. Further understanding of how the FADD-DD pathway works and why it is not able to work in immortal cells should provide new insights into the role of apoptosis and autophagy dysfunction in the development of epithelial cancers.

ACKNOWLEDGMENTS

We are grateful to Drs. R. Weinberg, W. Hahn, and B. Elenbaas for providing genetically defined human breast cells and Drs. T. Yoshimori and N. Mizushima for providing GFP-LC3. We thank Bilinda Dawson and Paula Moore for assistance with TEM. This work was supported by U.S. Army breast and prostate cancer research programs grants DAMD17-02-1-0612 and DAMD17-03-1-0049.

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